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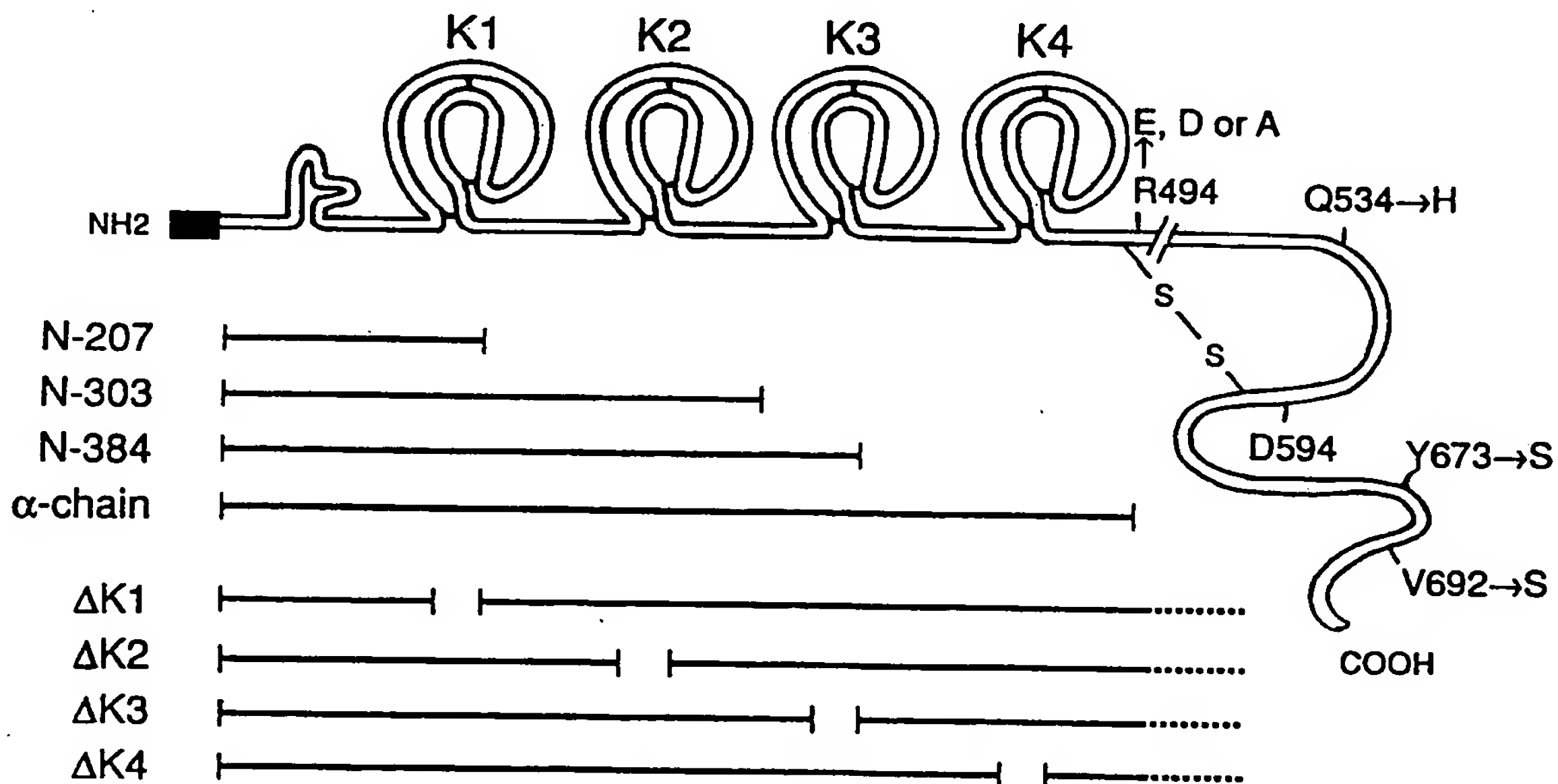
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(54) Title: **HEPATOCYTE GROWTH FACTOR VARIANTS**



(57) Abstract

The invention concerns hepatocyte growth factor (HGF) amino acid sequence variants. The preferred variants are resistant to proteolytic cleavage by enzymes capable of *in vivo* conversion of HGF into its two-chain form and/or contain a mutation within the protease domain of HGF.

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HEPATOCYTE GROWTH FACTOR VARIANTSBACKGROUND OF THE INVENTIONI. Field of the Invention

5 The present invention concerns amino acid sequence variants of hepatocyte growth factor (HGF), methods and means for preparing such variants, and pharmaceutical compositions comprising them.

II. Description of Background and Related Art

10 HGF was identified initially as a mitogen for hepatocytes [Michalopoulos *et al.*, Cancer Res. 44, 4414-4419 (1984); Russel *et al.*, J. Cell. Physiol. 119, 183-192 (1984) and Nakamura *et al.*, Biochem. Biophys. Res. Comm. 122:1450-1459 (1984)]. Nakamura *et al.*, *Supra* reported the purification of HGF from the serum of partially
15 hepatectomized rats. Subsequently, HGF was purified from rat platelets, and its subunit structure was determined [Nakamura *et al.*, Proc. Natl. Acad. Sci. USA, 83, 6489-6493 (1986); and Nakamura *et al.*, FEBS Letters 224, 311-316 (1987)]. The purification of human HGF (huHGF) from human plasma was first described by Gohda *et al.*, J. Clin. Invest. 81, 414-419 (1988).
20

 Both rat HGF and huHGF have been molecularly cloned, including the cloning and sequencing of a naturally occurring variant lacking 5 amino acids designated "delta5 HGF" [Miyazawa *et al.*, Biochem. Biophys. Res. Comm. 163, 967-973 (1989); Nakamura *et al.*, Nature 342, 440-443 (1989); Seki *et al.*, Biochem. and Biophys. Res. Commun. 172, 321-327 (1990); Tashiro *et al.*, Proc. Natl. Acad. Sci. USA 87, 3200-3204 (1990); Okajima *et al.*, Eur. J. Biochem. 193, 375-381 (1990)].
25

 The mature form of huHGF, corresponding to the major form purified from human serum, is a disulfide linked heterodimer derived
30 by proteolytic cleavage of the human pro-hormone between amino acids R494 and V495. This cleavage process generates a molecule composed of an α -subunit of 440 amino acids (M_r 69 kDa) and a β -subunit of 234 amino acids (M_r 34 kDa). The nucleotide sequence of the hHGF cDNA reveals that both the α - and the β -chains are contained in a single
35 open reading frame coding for a pre-pro precursor protein. In the predicted primary structure of mature hHGF, an interchain S-S bridge is formed between Cys 487 of the α -chain and Cys 604 in the β -chain (see Nakamura *et al.*, Nature, *supra*). The N-terminus of the α -chain

is preceded by 54 amino acids, starting with a methionine group. This segment includes a characteristic hydrophobic leader (signal) sequence of 31 residues and the prosequence. The α -chain starts at amino acid (aa) 55, and contains four Kringle domains. The so called "hairpin domain" includes amino acid residues 70-96 of wild-type human HGF. The Kringle 1 domain extends from about aa 128 to about aa 206, the Kringle 2 domain is between about aa 211 and about aa 288, the Kringle 3 domain is defined as extending from about aa 303 to about aa 383, and the Kringle 4 domain extends from about aa 391 to about aa 464 of the α -chain. It will be understood that the definition of the various Kringle domains is based on their homology with kringle-like domains of other proteins (prothrombin, plasminogen), therefore, the above limits are only approximate. As yet, the function of these Kringles has not been determined. The β -chain of huHGF shows high homology to the catalytic domain of serine proteases (38% homology to the plasminogen serine protease domain). However, two of the three residues which form the catalytic triad of serine proteases are not conserved in huHGF. Therefore, despite its serine protease-like domain, hHGF appears to have no proteolytic activity and the precise role of the β -chain remains unknown. HGF contains four putative glycosylation sites, which are located at positions 294 and 402 of the α -chain and at positions 566 and 653 of the β -chain.

In a portion of cDNA isolated from human leukocytes in-frame deletion of 15 base pairs was observed. Transient expression of the cDNA sequence in COS-1 cells revealed that the encoded HGF molecule (delta5 HGF) lacking 5 amino acids in the Kringle 1 domain was fully functional (Seki *et al.*, *supra*).

A naturally occurring huHGF variant has recently been identified which corresponds to an alternative spliced form of the huHGF transcript containing the coding sequences for the N-terminal finger and first two kringle domains of mature huHGF [Chan *et al.*, *Science* 254, 1382-1385 (1991); Miyazawa *et al.*, *Eur. J. Biochem.* 197, 15-22 (1991)]. This variant, designated HGF/NK2, has been proposed to be a competitive antagonist of mature huHGF.

The comparison of the amino acid sequence of rat HGF with that of huHGF revealed that the two sequences are highly conserved and have the same characteristic structural features. The length of the four Kringle domains in rat HGF is exactly the same as in huHGF.

Furthermore, the cysteine residues are located in exactly the same positions; an indication of similar three-dimensional structures (Okajima *et al.*, *supra*; Tashiro *et al.*, *supra*).

5 The HGF receptor has been identified as the product of the c-Met proto-oncogene [Bottaro *et al.*, *Science* **251**, 802-804 (1991); Naldini *et al.*, *Oncogene* **6**, 501-504 (1991)], an 190-kDa heterodimeric (a disulfide-linked 50-kDa α -chain and a 145-kDa β -chain) membrane-spanning tyrosine kinase protein [Park *et al.*, *Proc. Natl. Acad. Sci. USA* **84**, 6379-6383 (1987)]. The c-Met protein becomes phosphorylated on
10 tyrosine residues of the 145-kDa β -subunit upon HGF binding.

The levels of HGF increase in the plasma of patients with hepatic failure (Gohda *et al.*, *supra*) and in the plasma [Lindroos *et al.*, *Hepatol.* **13**, 734-750 (1991)] or serum [Asami *et al.*, *J. Biochem.* **109**, 8-13 (1991)] of animals with experimentally induced liver damage. The
15 kinetics of this response is rapid, and precedes the first round of DNA synthesis during liver regeneration suggesting that HGF may play a key role in initiating this process. More recently, HGF has been shown to be a mitogen for a variety of cell types including melanocytes, renal tubular cells, keratinocytes, certain endothelial
20 cells and cells of epithelial origin [Matsumoto *et al.*, *Biochem. Biophys. Res. Commun.* **176**, 45-51 (1991); Igawa *et al.*, *Biochem. Biophys. Res. Commun.* **174**, 831-838 (1991); Han *et al.*, *Biochem.* **30**, 9768-9780 (1991); Rubin *et al.*, *Proc. Natl. Acad. Sci. USA* **88**, 415-419 (1991)]. Interestingly, HGF can also act as a "scatter factor", an
25 activity that promotes the dissociation of epithelial and vascular endothelial cells in vitro [Stoker *et al.*, *Nature* **327**, 239-242 (1987); Weidner *et al.*, *J. Cell Biol.* **111**, 2097-2108 (1990); Naldini *et al.*, *EMBO J.* **10**, 2867-2878 (1991)]. Moreover, HGF has recently been described as an epithelial morphogen [Montesano *et al.*, *Cell* **67**, 901-
30 908 (1991)]. Therefore, HGF has been postulated to be important in tumor invasion and in embryonic development. Chronic c-Met/HGF receptor activation has been observed in certain malignancies [Cooper *et al.*, *EMBO J.* **5**, 2623 (1986); Giordano *et al.*, *Nature* **339**, 155 (1989)].

35 It would be desirable to better understand the structure-activity relationship of HGF in order to identify functionally important domains in the HGF amino acid sequence.

It would be particularly desirable to identify the amino acid residues which are responsible for the interaction of HGF with its receptor.

5 It would be also desirable to identify the amino acid residues which are responsible for HGF biological activity.

It would further be desirable to provide amino acid sequence variants of HGF that have altered (preferably enhanced) receptor binding affinity as compared to the corresponding mature, wild-type HGF.

10 It would also be desirable to provide HGF amino acid sequence variants which have retained or enhanced receptor binding affinity as compared to the corresponding wild-type HGF, but are substantially devoid of HGF biological activity. Such molecules could act as competitive antagonists of HGF action.

15 It would further be desirable to provide HGF amino acid sequence variants that have retained or enhanced receptor binding affinity and increased biological activity as compared to the corresponding wild-type HGF (HGF agonists). Accordingly, it is an object of the present invention to provide HGF variants having retained or improved
20 the receptor binding affinity of the corresponding mature wild-type HGF. It is another object of the invention to provide HGF variants that have retained substantially full receptor binding affinity of the corresponding mature wild-type HGF and are substantially incapable of HGF receptor activation. It is a further
25 object to provide HGF variants that have retained substantially full receptor binding affinity of the corresponding mature wild-type HGF and have improved biological properties.

These and further objects will be apparent to one of ordinary skill in the art.

30

SUMMARY OF THE INVENTION

The foregoing objects are achieved by the provision of HGF variants having amino acid alterations within various domains of the wild-type HGF amino acid sequence.

35 In one aspect, HGF variants are provided that are resistant to proteolytic cleavage by enzymes that are capable of in vivo conversion of HGF into its two-chain form. The variants are preferably stabilized in single-chain form by site directed mutagenesis within a

region recognized by an enzyme capable of converting HGF into its two-chain form.

In a particular embodiment, such variants have an amino acid alteration at or adjacent to amino acid positions 493, 494, 495 or 496 of the wild-type huHGF amino acid sequence. The alteration preferably is the substitution of at least one amino acid at amino acid positions 493-496 of the wild-type huHGF amino acid sequence.

In another embodiment, the variants retain substantially full receptor binding affinity of the corresponding wild-type HGF and are substantially incapable of HGF receptor activation. HGF variants with enhanced receptor binding affinity and substantially lacking the ability to activate the HGF receptor are particularly preferred. Such compounds are competitive antagonists of the corresponding wild-type HGF and, when present in sufficient concentration, are capable of inhibiting the binding of their wild-type counterparts to their ligands.

In another aspect, here are provided HGF variants having an amino acid alteration at a site within the protease domain of HGF and retaining substantially full receptor binding affinity of the corresponding wild-type HGF.

In a specific embodiment, these variants have substantially retained or improved receptor binding affinity as compared to the corresponding wild-type HGF, and are substantially devoid of HGF biological activity. Such compounds, if present in sufficient concentration, will act as competitive antagonists of HGF action.

In another specific embodiment, the variants combine substantially retained or improved receptor binding affinity with improved biological activity, as compared to the corresponding wild-type HGF. Such variants are valuable as HGF agonists.

In a preferred embodiment, the HGF variants within this group comprise an alteration in a region corresponding to the catalytic site of serine proteases. More preferably the alteration is at or adjacent to any of positions 534, 673 and 692 of the wild-type human HGF (huHGF) amino acid sequence.

The alteration preferably is substitution.

In a particularly preferred embodiment, at least two of the residues at amino acid positions 534, 673 and 692 of the wild-type huHGF sequence are replaced by another amino acid.

In a preferred group of the HGF variants herein, both tyrosine (Y) at position 673 and valine (V) at position 692 of the huHGF sequence are replaced by another amino acid. This alteration potentially yields HGF variants which substantially retain the receptor binding affinity of wild-type huHGF but are substantially devoid of HGF biological activity.

The mutations around the one-chain to two-chain cleavage site and within the protease domain may be advantageously combined for improved biological properties.

Variants with increased receptor binding affinity as compared to the corresponding wild-type HGF are particularly preferred. The increase in receptor binding affinity may, for example, be accomplished by an alteration in the receptor-binding domain of the wild-type HGF amino acid sequence, and preferably within the Kringle 1 domain.

Kringle 1 variants with amino acid alterations within the patch defined by amino acid positions 159, 161, 195 and 197, or at amino acid position 173 of the wild-type huHGF amino acid sequence are particularly preferred, but other positions within the Kringle 1 domain have also been identified as having a genuine effect on the receptor binding properties and/or the specific activity of HGF.

Furthermore, amino acid sequence variants with alterations at amino acid positions preceding the Kringle 1 domain, in particular those just N- or C-terminal to the hairpin domain, have been found to have significantly different binding properties and biological activity from those of the corresponding wild-type HGF.

The variants of this invention may be devoid of functional Kringle 2 and/or Kringle 3 and/or Kringle 4 domains.

In all embodiments, huHGF amino acid sequence variants are preferred.

In other embodiments, the invention relates to DNA sequences encoding the variants described above, replicable expression vectors containing and capable of expressing such DNA sequences in a transformed host cell, transformed host cells, and a process comprising culturing the host cells so as to express the DNAs encoding the HGF variants.

In yet another embodiment, the invention relates to therapeutic compositions comprising HGF variants having HGF agonist or antagonist properties.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 is a schematic representation of the α - and β -subunits of huHGF. Shown in the α -chain are the signal sequence (boxed region) which encompasses amino acids 1 - 31, the predicted finger and four Kringle domains, each with their respective three disulfide bonds. The cleavage site for generation of the heterodimeric α/β form of huHGF
10 immediately follows the P1 cleavage residue R494. This last residue has been specifically substituted with either E, D or A to generate HGF single-chain variants. The β -chain, which follows the cleavage site, contains homology to serine proteases. It is proposed that the α - and β -chains are held together by a unique disulfide-bridge between
15 C487(α) and C604(β) (Nakamura et al., 1989, supra). Three residues within the β -chain have been substituted individually or in combination to reconstitute the authentic residues of a serine-protease. Schematic representations of the mature forms of the C-terminal truncation variants are depicted below: N-207, deleted after
20 the first Kringle; N-303, deleted after the second Kringle; N-384, deleted after the third Kringle and the α -chain. Also shown are the variants where deletions of each of the Kringles ($\Delta K1$, $\Delta K2$, $\Delta K3$ and $\Delta K4$) were introduced. In each case, the deletions specifically remove the entire Kringle from C1 to C6.

25 Figure 2 shows the results of Western blot of wild-type rhuHGF and single-chain variants. Conditioned media from mock transfected 293 cells or stable 293 cells expressing either wild-type rhuHGF (WT) or the variants R494E, R494A or R494D were fractionated under reducing conditions on an 8% sodium-dodecyl sulfate-polyacrylamide gel and
30 blotted. The blot was reacted with polyclonal anti-HGF antisera which recognizes epitopes primarily in the α -chain. Molecular masses (kilodaltons) of the marker are as indicated. Also indicated are the positions of the α -chain and uncleaved single-chain forms of huHGF. Note that the polyclonal antibody cross-reacts with an unidentified
35 band (*) present even in the control transfected 293 cells, which do not express detectable quantities of huHGF.

Figure 3: Mitogenic activity (A) and competitive receptor binding (B) of wild-type (WT) rhuHGF and single-chain variants. (A) Biological

activity was determined by the ability of WT rhuHGF and variants to induce DNA synthesis of rat hepatocytes in primary culture as described in Example 2. Shown are the mean cpm from duplicates in a representative assay. Mock supernatant from control cells did not stimulate DNA synthesis in these cells (no cpm increase above background levels). (B) To perform competitive binding, various dilutions of supernatants of human 293 cells containing wt rhuHGF or variants were incubated with 50 pM of the huHGF receptor-IgG fusion protein as described in Example 2. Data represent inhibition of binding as the percentage of any competing ligand from a representative experiment and were corrected by subtraction of background values from control 293 cells.

Figure 4: Western blot of ligand-induced tyrosine-phosphorylation on the 145 kDa β -subunit of the HGF receptor by wild-type rhuHGF, single-chain or protease domain huHGF variants. Lysates from A549 cells incubated for 5 minutes without (-) or with 200 ng/mL of purified wt rhuHGF (WT), single-chain (R494E) or double protease variants (Y673S,V692S) were prepared and immunoprecipitated with an anti-HGF receptor antibody and blotted with anti-phosphotyrosine antibodies. Molecular masses (kilodaltons) are as indicated.

Figure 5 depicts the nucleotide sequence encoding the plasmid pRK5.1 (SEQ. ID. NO: 1).

Figure 6 depicts the nucleotide sequence encoding the plasmid p.CIS.EBON (SEQ. ID. NO: 15).

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

As used herein, the terms "hepatocyte growth factor", "HGF" and "huHGF" refer to a (human) growth factor capable of specific binding to a receptor of wild-type (human) HGF, which growth factor typically has a structure with six domains (finger, Kringle 1, Kringle 2, Kringle 3, Kringle 4 and serine protease domains), but nonetheless may have fewer domains or may have some of its domains repeated if it still retains its qualitative HGF receptor binding ability. This definition specifically includes the delta5 huHGF as disclosed by Seki *et al.*, *supra*. The terms "hepatocyte growth factor" and "HGF" also include hepatocyte growth factor from any non-human animal species, and in particular rat HGF.

The terms "wild-type human hepatocyte growth factor", "native human hepatocyte growth factor", "wild-type huHGF", and "native huHGF" refer to native sequence human HGF, i.e., that encoded by the cDNA sequence published by Miyazawa, et al. 1989, supra, or Nakamura et al., 1989, supra, including its mature, pre, pre-pro, and pro forms, purified from natural source, chemically synthesized or recombinantly produced. The sequences reported by Miyazawa et al. and Nakamura et al. differ in 14 amino acids. The reason for the differences is not entirely clear; polymorphism or cloning artifacts are among the possibilities. Both sequences are specifically encompassed by the foregoing terms as defined for the purpose of the present invention. It will be understood that natural allelic variations exist and can occur among individuals, as demonstrated by one or more amino acid differences in the amino acid sequence of each individual. Amino acid positions in the variant huHGF molecules herein are indicated in accordance with the numbering of Miyazawa et al. 1989, supra.

The terms "(HGF) biological activity", "biologically active", "activity" and "active" refer to any mitogenic, motogenic or morphogenic activities exhibited by wild-type human HGF. The HGF biological activity may, for example, be determined in an in vitro or in vivo assay of hepatocyte growth promotion. Adult rat hepatocytes in primary culture have been extensively used to search for factors that regulate hepatocyte proliferation. Accordingly, the mitogenic effect of an HGF variant can be conveniently determined in an assay suitable for testing the ability of an HGF molecule to induce DNA synthesis of rat hepatocytes in primary cultures, such as, for example, described in Example 2. Human hepatocytes are also available from whole liver perfusion on organs deemed unacceptable for transplantation, pare-downs of adult livers used for transplantation in children, fetal livers and liver remnants removed at surgery for other indications. Human hepatocytes can be cultured similarly to the methods established for preparing primary cultures of normal rat hepatocytes. Hepatocyte DNA synthesis can, for example, be assayed by measuring incorporation of [³H]thymidine into DNA, with appropriate hydroxyurea controls for replicative synthesis.

The effect of HGF variants on hepatocyte growth can also be tested in vivo in animal models of liver dysfunction and regeneration, such as in rats following partial hepatectomy, or carbon tetrachloride

caused hepatic injury, in D-galactosamine induced acute liver failure models, etc. According to a suitable protocol, a liver poison, e.g. α -naphthylisothiocyanate (ANIT) is administered to rats in a predetermined concentration capable of causing reproducible significant elevation of liver enzyme and bilirubin levels. The rats are then treated with the HGF variant to be tested, sacrificed and the liver enzyme and bilirubin levels are determined. The livers are additionally observed for hepatic lesions.

The expression "retaining substantially full receptor binding affinity of wild-type (hu)HGF" and grammatical variant thereof as used herein mean that the receptor binding affinity of the HGF variant is not less than about 70%; preferably not less than about 80%, more preferably not less than about 90%, most preferably not less than about 95% of the affinity with which wild-type (hu)HGF binds its native receptor.

The terms "substantially incapable of HGF receptor activation" and "substantially devoid of HGF biological activity" mean that the activity exhibited by an HGF variant is less than about 20%, preferably less than about 15%, more preferably less than about 10%, most preferably less than about 5% of the respective activity of wild-type (human) HGF in an established assay of receptor activation or HGF biological activity, as hereinabove defined.

The terms "amino acid" and "amino acids" refer to all naturally occurring L- α -amino acids. This definition is meant to include norleucine, ornithine, and homocysteine. The amino acids are identified by either the single-letter or three-letter designations:

Asp	D	aspartic acid	Ile	I	isoleucine
Thr	T	threonine	Leu	L	leucine
Ser	S	serine	Tyr	Y	tyrosine
Glu	E	glutamic acid	Phe	F	phenylalanine
Pro	P	proline	His	H	histidine
Gly	G	glycine	Lys	K	lysine
Ala	A	alanine	Arg	R	arginine
Cys	C	cysteine	Trp	W	tryptophan
Val	V	valine	Gln	Q	glutamine
Met	M	methionine	Asn	N	asparagine

These amino acids may be classified according to the chemical composition and properties of their side chains. They are broadly

classified into two groups, charged and uncharged. Each of these groups is divided into subgroups to classify the amino acids more accurately:

I. Charged Amino Acids

5 Acidic Residues: aspartic acid, glutamic acid

Basic Residues: lysine, arginine, histidine

II. Uncharged Amino Acids

Hydrophilic Residues: serine, threonine, asparagine,
 glutamine

10 Aliphatic Residues: glycine, alanine, valine, leucine,
 isoleucine

Non-polar Residues: cysteine, methionine, proline

Aromatic Residues: phenylalanine, tyrosine, tryptophan

 The terms "alteration", "amino acid alteration", "variant" and
15 "amino acid sequence variant" refer to HGF molecules with some
differences in their amino acid sequences as compared to wild-type
(human) HGF. Ordinarily, the variants will possess at least about 80%
homology with those domains of wild-type (human) HGF that are retained
in their structure, and preferably, they will be at least about 90%
20 homologous with such domains.

 Substitutional HGF variants are those that have at least one
amino acid residue in the corresponding wild-type HGF sequence removed
and a different amino acid inserted in its place at the same position.
The substitutions may be single, where only one amino acid in the
25 molecule has been substituted, or they may be multiple, where two or
more amino acids have been substituted in the same molecule.

 Substantial changes in the activity of the HGF molecule may be
obtained by substituting an amino acid with a side chain that is
significantly different in charge and/or structure from that of the
30 native amino acid. This type of substitution would be expected to
affect the structure of the polypeptide backbone and/or the charge or
hydrophobicity of the molecule in the area of the substitution.

 Moderate changes in the activity of the HGF molecule would be
expected by substituting an amino acid with a side chain that is
35 similar in charge and/or structure to that of the native molecule.
This type of substitution, referred to as a conservative substitution,
would not be expected to substantially alter either the structure of

the polypeptide backbone or the charge or hydrophobicity of the molecule in the area of the substitution.

Insertional HGF variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in the wild-type HGF molecule. Immediately adjacent to an amino acid means connected to either the α -carboxy or α -amino functional group of the amino acid. The insertion may be one or more amino acids. Ordinarily, the insertion will consist of one or two conservative amino acids. Amino acids similar in charge and/or structure to the amino acids adjacent to the site of insertion are defined as conservative. Alternatively, this invention includes insertion of an amino acid with a charge and/or structure that is substantially different from the amino acids adjacent to the site of insertion.

Deletional variants are those with one or more amino acids in the wild-type HGF molecule removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the HGF molecule.

The notations used throughout this application to describe huHGF amino acid sequence variants are described below. The location of a particular amino acid in the polypeptide chain of huHGF is identified by a number. The number refers to the amino acid position in the amino acid sequence of the mature, wild-type human HGF polypeptide as disclosed in Miyazawa *et al.*, 1989, *supra*. In the present application, similarly positioned residues in huHGF variants are designated by these numbers even though the actual residue number is not so numbered due to deletions or insertions in the molecule. This will occur, for example, with site-directed deletional or insertional variants. The amino acids are identified using the one-letter code. Substituted amino acids are designated by identifying the wild-type amino acid on the left side of the number denoting the position in the polypeptide chain of that amino acid, and identifying the substituted amino acid on the right side of the number.

For example, replacement of the amino acid arginine (R) by glutamic acid (E) at amino acid position 494 of the wild-type huHGF molecule yields a huHGF variant designated R494E huHGF. Similarly, the huHGF variant obtained by substitution of serine (S) for tyrosine (Y) at amino acid position 673 and serine (S) for valine (V) at amino

acid position 692 of the wild-type huHGF molecule is designated Y673S,V692S huHGF.

Deletional variants are identified by indicating the amino acid residue and position at either end of the deletion, inclusive, and placing the Greek letter delta, " Δ ", to the left of the indicated amino acids. Deletion of a single amino acid is indicated by placing Δ to the left of the single letter code and number indicating the position of the deleted amino acid.

Insertional variants are designated by the use of brackets "[]" around the inserted amino acids, and the location of the insertion is denoted by indicating the position of the amino acid on either side of the insertion.

The alterations in the amino acid sequence of the HGF variants herein are indicated with reference to amino acid positions in the wild-type human HGF amino acid sequence. (Miyazawa et al., supra). Methods for the alignment of homologous amino acid sequences from various species are well known in the art.

The terms "DNA sequence encoding", "DNA encoding" and "nucleic acid encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide chain. The DNA sequence thus codes for the amino acid sequence.

The terms "replicable expression vector" and "expression vector" refer to a piece of DNA, usually double-stranded, which may have inserted into it a piece of foreign DNA. Foreign DNA is defined as heterologous DNA, which is DNA not naturally found in the host cell. The vector is used to transport the foreign or heterologous DNA into a suitable host cell. Once in the host cell, the vector can replicate independently of the host chromosomal DNA, and several copies of the vector and its inserted (foreign) DNA may be generated. In addition, the vector contains the necessary elements that permit translating the foreign DNA into a polypeptide. Many molecules of the polypeptide encoded by the foreign DNA can thus be rapidly synthesized.

In the context of the present invention the expressions "cell", "cell line", and "cell culture" are used interchangeably, and all such designations include progeny.

The terms "transformed (host) cell" , "transformant" and "transformed" refer to the introduction of DNA into a cell. The cell is termed a "host cell". The introduced DNA is usually in the form of a vector containing an inserted piece of DNA. The introduced DNA
5 sequence may be from the same species as the host cell or a different species from the host cell, or it may be a hybrid DNA sequence, containing some foreign and some homologous DNA. The words transformants and transformed (host) cells include the primary subject cell and cultures derived therefrom, without regard to the number of
10 transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological property as screened for in the originally transformed cell are included.

15 The technique of "polymerase chain reaction" or "PCR", as used herein, generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195, issued 28 July 1987 and in Current Protocols in Molecular Biology, Ausubel et al. eds., Greene
20 Publishing Associates and Wiley-Interscience 1991, Volume 2, Chapter 15.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population
25 are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. The monoclonal antibodies include hybrid and recombinant antibodies produced by splicing a variable (including
30 hypervariable) domain of an anti-selectin ligand antibody with a constant domain (e.g. "humanized" antibodies), only one of which is directed against a selectin, or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or
35 immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv). Cabilly, et al., U.S. Pat. No. 4,816,567; Mage & Lamoyi, in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc., New York,

1987). Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from such a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method.

5 The term "immunoglobulin" generally refers to polypeptides comprising a light or heavy chain usually both disulfide bonded in the native "Y" configuration, although other linkage between them, including tetramers or aggregates thereof, is within the scope hereof.

10 Immunoglobulins (Ig) and certain variants thereof are known and many have been prepared in recombinant cell culture. For example, see U.S. Patent 4,745,055; EP 256,654; Faulkner *et al.*, Nature 298:286 (1982); EP 120,694; EP 125,023; Morrison, J. Immun. 123:793 (1979); Köhler *et al.*, Proc. Nat'l. Acad. Sci. USA 77:2197 (1980); Raso *et*
15 *al.*, Cancer Res. 41:2073 (1981); Morrison *et al.*, Ann. Rev. Immunol. 2:239 (1984); Morrison, Science 229:1202 (1985); Morrison *et al.*, Proc. Nat'l. Acad. Sci. USA 81:6851 (1984); EP 255,694; EP 266,663; and WO 88/03559. Reassorted immunoglobulin chains also are known. See for example U.S. patent 4,444,878; WO 88/03565; and EP 68,763 and
20 references cited therein. The immunoglobulin moiety in the chimeras of the present invention may be obtained from IgG₁, IgG₂, IgG₃, or IgG₄ subtypes, IgA, IgE, IgD or IgM, but preferably IgG₁ or IgG₃.

II. Selection of the HGF Variants

25 The present invention is based upon the study of structure-activity and structure-receptor binding relationship in amino acid sequence variants of HGF.

Certain HGF variants of the present invention are resistant to proteolytic cleavage by enzymes that are capable of in vivo conversion
30 of the single-chain HGF proenzyme into its two-chain form. Such enzymes are trypsin-like proteases. Absent alterations, the proteolytic cleavage takes place between Arg494 and Val495 of the wild-type huHGF sequence. The resistance to proteolytic cleavage is preferably achieved by site-directed mutagenesis within a region
35 recognized by an enzyme capable of converting HGF into its two-chain form, and preferably within the Leu-Arg-Val-Val (LRVV) sequence at amino acid residues 493-496 of the wild-type huHGF sequence. The variants herein may, for example, contain single or multiple amino

acid substitutions, insertions or deletions at or adjacent to amino acid positions 493, 494, 495 and 496 in the wild-type human HGF amino acid sequence.

5 A preferred alteration is the replacement of arginine at amino acid position 494 with any other amino acid, preferably glutamic acid, aspartic acid or alanine. In general, the substitution of smaller, apolar or acidic amino acids for arginine at this position is believed to yield single-chain HGF variants.

10 Alternatively or in addition, the replacement of valine at position 495 by another amino acid is expected to block the one-chain to two-chain cleavage. Bulkier amino acids, such as tyrosine, phenylalanine, etc. are preferred for substitution at this position.

15 Other HGF variants of the present invention are altered at a site within the protease domain of HGF and retain substantially full receptor binding affinity of the corresponding (preferably human) wild-type HGF. The protease domain follows the cleavage site between amino acid positions 494 and 495 in the wild-type huHGF sequence, and shows a high degree of homology with the catalytic domain of known serine proteases. The conservation does not apply to
20 the active site of serine proteases. In human plasmin, which is formed from its proenzyme, plasminogen, residues His-42, Asp-85 and Ser-181 form the catalytic site (catalytic triad). This catalytic triad is highly conserved in serine proteases. In the huHGF amino acid sequence asparagine is retained at amino acid position 594,
25 however, position 534 (corresponding to position 42 of plasmin) is occupied by glutamine instead of histidine, and position 673 (corresponding to position 181 of plasmin) by tyrosine instead of serine. A preferred group of the protease domain alterations herein involves one or both of amino acid positions 673 and 534.
30 Alternatively, or in addition, the alteration may be at position 692 of the huHGF amino acid sequence. In all instances, the alteration preferably is the substitution of one or more different amino acids for the residues at these positions of the native huHGF amino acid sequence.

35 Tyrosine at amino acid position 673 is preferably replaced by an amino acid which has no bulky aromatic or heterocyclic moieties. Such amino acids include serine, threonine, asparagine, cysteine, glycine,

alanine and valine. In the preferred variants, serine is substituted for tyrosine at this position.

Valine at amino acid position 692 preferably is substituted by a polar amino acid, such as serine, threonine, asparagine or glutamine, preferably serine.

In a preferred group of the HGF amino acid sequence variants herein, both position 673 and position 692 are substituted by one of the foregoing amino acids, preferably serine. Such variants may additionally contain an alteration (preferably substitution) at amino acid position 534. The latter alteration may be the substitution of histidine for glutamine in the wild-type huHGF amino acid sequence.

The single, double or triple mutations within the protease domain may be combined with additional alterations in the wild-type HGF amino acid sequence. Such further alterations may, for example, be at or around the one-chain to two chain cleavage site of the HGF molecule, as hereinabove described, and may result in variants which are substantially in single-chain form.

Additional alterations may be at the C-terminal end and/or in the Kringle domains of the HGF molecule. In addition to the deletion mutants disclosed in the examples, HGF variants with alterations within the Kringle 1 domain are of great interest. As we have found that the receptor binding domain is contained within the finger and the Kringle 1 regions of the HGF molecule, amino acid alterations within these domains are expected to significantly alter the receptor binding properties (and the biological activity) of the variants of the present invention. Alterations at residues that are most exposed to the interior in the Kringle structure (mostly charged residues) are particularly likely to cause profound changes in the receptor binding properties and/or biological activity of the HGF variants.

Alterations within the Kringle 1 domain preferably are within the patch defined by amino acid positions 159, 161, 195 and 197 of the wild-type huHGF amino acid sequence or at corresponding positions in a non-human HGF amino acid sequence. Another preferred site for amino acid alteration is at position 173 of the wild-type huHGF amino acid sequence. The latter position is at the opposite side as compared to the surface defined by amino acid position 159, 161, 195 and 197 and the reasons for its involvement in the binding properties and biological activity of HGF have not yet been fully identified.

Some illustrative huHGF variants within the scope herein are as follows: R494E; R494D; R494A; V495Y; V495F; R494E, V495Y; R494E, V495F; R494D, V495Y; R494D, V495F; R494A, V495Y; R494A, V495F; R494[E]V495; R494[D]V495; R494[A]V495; R494[Y]V495; R494[F]V495; R494E, Q534H; R494E, Y673S; R494E, V692S; R494D, Q534H; R494D, Y673S; R494D, V692S, R494A, Q534H; R494A, V673S; R494A, V692S, R494E, Y673S, V692S; R494D, Y673S, V692S, R494A, Y673S, V692S, R494E, Q534H, Y673S, V692S; R494D, Q534H, Y673S, V692S; R494A, Q534H, Y673S, V692S; E159A; S161A; F162A, L163A, S165A, S166A; F162A; L163A, S165A, S166A; Y167F; Y167A; R168A; Q173A; Q173A, E174A, N175A; N193A; R195A; R197A; N193A, E195A, R197A; K52A; D54A; K52A, D54A; H114A; H114A, E115A, D117A; E115A; D117A; variants with combinations of any of the foregoing alterations; Δ K3 and/or Δ K4 variants comprising any of the foregoing alterations; corresponding delta5-huHGF variants and non-human animal HGF variants.

III. Construction of the HGF Variants

Whereas any technique known in the art can be used to perform site-directed mutagenesis, e.g. as disclosed in Sambrook et al. [Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press, New York (1989)], oligonucleotide-directed mutagenesis is the preferred method for preparing the HGF variants of this invention. This method, which is well known in the art [Adelman et al. DNA, 2:183 (1983), Sambrook et al., Supra], is particularly suitable for making substitution variants, it may also be used to conveniently prepare deletion and insertion variants.

As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing et al., Third Cleveland Symposium on Macromolecules and Recombinant DNA, Editor A. Walton, Elsevier, Amsterdam (1981). These phage are readily commercially available and their use is generally well known to those skilled in the art. Alternatively, plasmid vectors that contain a single-stranded phage origin of replication (Veira et al., Meth. Enzymol., 153: 3 (1987)) may be employed to obtain single-stranded DNA.

The oligonucleotides are readily synthesized using techniques well known in the art such as that described by Crea et al. (Proc. Nat'l. Acad. Sci. USA, 75:5765 [1978]).

5 The specific mutagenesis method followed in making the HGF variants of Example 1 was described by Kunkel et al., Methods in Enzymol. 154 367-382 (1987).

10 Mutants with more than one amino acid substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If however, the amino acids are located some distance from each other (separated by more than ten amino acids, for example) it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one
15 of two alternative methods may be employed. In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired
20 amino acid substitutions. The alternative method involves two or more rounds of mutagenesis to produce the desired mutant.

Another method for making mutations in the DNA sequence encoding wild-type HGF or a variant molecule known in the art, involves
25 cleaving the DNA sequence encoding the starting HGF molecule at the appropriate position by digestion with restriction enzymes, recovering the properly cleaved DNA, synthesizing an oligonucleotide encoding the desired amino acid sequence and flanking regions such as polylinkers with blunt ends (or, instead of polylinkers, digesting the synthetic oligonucleotide with the restriction enzymes also used to cleave the
30 HGF encoding DNA, thereby creating cohesive termini), and ligating the synthetic DNA into the remainder of the HGF encoding structural gene.

PCR mutagenesis is also suitable for making the HGF variants of the present invention, for example, as described in U.S. Patent No. 4,683,195 issued 28 July 1987 and in Current Protocols in Molecular
35 Biology, Ausubel et al., eds. Greene Publishing Associates and Wiley-Interscience, Volume 2, Chapter 15, 1991. While the following discussion refers to DNA, it is understood that the technique also find application with RNA. The PCR technique generally refers to the

following procedure. When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone. If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

The cDNA encoding the HGF variants of the present invention is inserted into a replicable vector for further cloning or expression.

Suitable vectors are prepared using standard recombinant DNA procedures. Isolated plasmids and DNA fragments are cleaved, tailored, and ligated together in a specific order to generate the desired vectors.

After ligation, the vector with the foreign gene now inserted is transformed into a suitable host cell. The transformed cells are selected by growth on an antibiotic, commonly tetracycline (tet) or ampicillin (amp), to which they are rendered resistant due to the presence of tet and/or amp resistance genes on the vector. If the ligation mixture has been transformed into a eukaryotic host cell,

transformed cells may be selected by the DHFR/MTX system. The transformed cells are grown in culture and the plasmid DNA (plasmid refers to the vector ligated to the foreign gene of interest) is then isolated. This plasmid DNA is then analyzed by restriction mapping and/or DNA sequencing. DNA sequencing is generally performed by either the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxam et al., Methods of Enzymology, 65:499 (1980).

Prokaryotes are the preferred host cells for the initial cloning steps of this invention. They are particularly useful for rapid production of large amounts of DNA, for production of single-stranded DNA templates used for site-directed mutagenesis, for screening many mutants simultaneously, and for DNA sequencing of the mutants generated. For expressing the HGF variants of the present invention eukaryotic hosts, such as eukaryotic microbes (yeast) and multicellular organisms (mammalian cell cultures) may also be used. Examples of prokaryotes, e.g. E. coli, eukaryotic microorganisms and multicellular cell cultures, and expression vectors, suitable for use in producing the HGF variants of the present invention are, for example, those disclosed in WO 90/02798 (published 22 March 1990).

Cloning and expression methodologies are well known in the art and are, for example, disclosed in the foregoing published PCT patent application (WO 90/02798).

If mammalian cells are used as host cells, transfection generally is carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, Virology, 52: 546 (1978). However, other methods for introducing DNA into cells such as nuclear injection, electroporation, or protoplast fusion are also suitably used.

If yeast are used as the host, transfection is generally accomplished using polyethylene glycol, as taught by Hinnen, Proc. Natl. Acad. Sci. U.S.A., 75: 1929-1933 (1978).

If prokaryotic cells or cells that contain substantial cell wall constructions are used, the preferred method of transfection is calcium treatment using calcium as described by Cohen et al., Proc. Natl. Acad. Sci. (USA) 69: 2110 (1972), or more recently electroporation.

The HGF variant preferably is recovered from the culture medium as a secreted protein, although it also may be recovered from host cell lysates when directly expressed without a secretory signal. When the variant is expressed in a recombinant cell other than one of human origin, the variant is thus completely free of proteins of human origin. However, it is necessary to purify the variant from recombinant cell proteins in order to obtain preparations that are substantially homogeneous as to protein. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris.

The variant is then purified from contaminant soluble proteins, for example, by an appropriate combination of conventional chromatography methods, e.g. gel filtration, ion-exchange, hydrophobic interaction, affinity, immunoaffinity chromatography, reverse phase HPLC; precipitation, e.g. ethanol precipitation, ammonium sulfate precipitation, or, preferably, immunoprecipitation with anti-HGF (polyclonal or monoclonal) antibodies covalently linked to Sepharose. Due to its high affinity to heparine, HGF can be conveniently purified on a heparin, such as heparine-Sepharose column. One skilled in the art will appreciate that purification methods suitable for native HGF may require modification to account for changes in the character of HGF or its variants upon expression in recombinant cell culture.

As hereinabove described, huHGF contains four putative glycosylation sites, which are located at positions 294 and 402 of the α -chain and at positions 566 and 653 of the β -chain. These positions are conserved in the rat HGF amino acid sequence. Glycosylation variants are within the scope herein.

Glycosylation of polypeptides is typically either N-linked or O-linked.. N-linked refers to the attachment of the carbohydrate moiety to the side-chain of an asparagine residue. The tripeptide sequences, asparagine-X-serine and asparagine-X-threonine, wherein X is any amino acid except proline, are recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be involved in O-linked glycosylation. O-linked glycosylation sites may, for example, be modified by the addition of, or substitution by, one or more serine or threonine

residue to the amino acid sequence of the HGF molecule. For ease, changes are usually made at the DNA level, essentially using the techniques discussed hereinabove with respect to the amino acid sequence variants.

5 Chemical or enzymatic coupling of glycosydes to the HGF variants of the present invention may also be used to modify or increase the number or profile of carbohydrate substituents. These procedures are advantageous in that they do not require production of the polypeptide that is capable of O-linked (or N-linked) glycosylation. Depending on
10 the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free hydroxyl groups such as those of cysteine, (d) free sulfhydryl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan or (f) the amide group
15 of glutamine. These methods are described in WO 87/05330 (published 11 September 1987), and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

 Carbohydrate moieties present on an HGF variant may also be removed chemically or enzymatically. Chemical deglycosylation
20 requires exposure to trifluoromethanesulfonic acid or an equivalent compound. This treatment results in the cleavage of most or all sugars, except the linking sugar, while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin et al., Arch. Biochem. Biophys. 259, 52 (1987) and by Edge et al., Anal. Biochem. 118, 131 (1981). Carbohydrate moieties can be removed by a
25 variety of endo- and exoglycosidases as described by Thotakura et al., Meth. Enzymol. 138, 350 (1987). Glycosylation is suppressed by tunicamycin as described by Duskin et al., J. Biol. Chem. 257, 3105 (1982). Tunicamycin blocks the formation of protein-N-glycosydase
30 linkages.

 Glycosylation variants of the amino acid sequence variants herein can also be produced by selecting appropriate host cells. Yeast, for example, introduce glycosylation which varies significantly from that of mammalian systems. Similarly, mammalian cells having a different
35 species (e.g. hamster, murine, insect, porcine, bovine or ovine) or tissue (e.g. lung, liver, lymphoid, mesenchymal or epidermal) origin than the source of the selectin variant, are routinely screened for the ability to introduce variant glycosylation. Covalent

modifications of an HGF variant molecule are included within the scope herein. Such modifications are traditionally introduced by reacting targeted amino acid residues of the HGF variant with an organic derivatizing agent that is capable of reacting with selected side-chains or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. The resultant covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays of the HGF variants, or for the preparation of anti-HGF antibodies for immunoaffinity purification of the recombinant glycoprotein. For example, complete inactivation of the biological activity of the protein after reaction with ninhydrin would suggest that at least one arginyl or lysyl residue is critical for its activity, whereafter the individual residues which were modified under the conditions selected are identified by isolation of a peptide fragment containing the modified amino acid residue. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

Derivatization with bifunctional agents is useful for preparing intramolecular aggregates of the HGF variants as well as for cross-linking the HGF variants to a water insoluble support matrix or surface for use in assays or affinity purification. In addition, a study of interchain cross-links will provide direct information on conformational structure. Commonly used cross-linking agents include 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, homobifunctional imidoesters, and bifunctional maleimides. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithiol]propioimide yield photoactivatable intermediates which are capable of forming cross-links in the presence of light. Alternatively, reactive water insoluble matrices such as cyanogen bromide activated carbohydrates and the systems reactive substrates described in U.S. patent Nos. 3,959,642; 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; 4,055,635; and 4,330,440 are employed for protein immobilization and cross-linking.

Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutamyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl

residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

5 Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)].

10 Other derivatives comprise the novel HGF variants of this invention covalently bonded to a nonproteinaceous polymer. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e. a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or in
15 vitro methods are useful, as are polymers which are isolated from nature. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyvinylalkylene ethers such a polyethylene glycol, polypropylene glycol.

20 The HGF variants may be linked to various nonproteinaceous polymers, such as polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

25 The HGF variants may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, in colloidal drug delivery systems (e.g. liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th Edition, Osol, A., Ed. (1980). An
30 HGF variant sequence can be linked to a immunoglobulin constant domain sequence as hereinbefore defined. The resultant molecules are commonly referred to as HGF variant-immunoglobulin chimeras. Such chimeras can be constructed essentially as described in WO 91/08298 (published 13 June 1991).

35 Ordinarily, the HGF variant is fused C-terminally to the N-terminus of the constant region of an immunoglobulin in place of the variable region(s), however N-terminal fusions of the selectin

variants are also desirable. The transmembrane regions of the HGF variants are preferably inactivated or deleted prior to fusion.

Typically, such fusions retain at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain. This ordinarily is accomplished by constructing the appropriate DNA sequence and expressing it in recombinant cell culture.

Alternatively, however, the HGF variant-immunoglobulin chimeras of this invention may be synthesized according to known methods.

The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics of the HGF variant.

In some embodiments, the hybrid immunoglobulins are assembled as monomers, or hetero- or homo-multimers, and particularly as dimers or tetramers, essentially as illustrated in WO 91/08298, Supra.

In a preferred embodiment, the C-terminus of a sequence which contains the binding site(s) for an HGF receptor, is fused to the N-terminus of the C-terminal portion of an antibody (in particular the Fc domain), containing the effector functions of an immunoglobulin, e.g. immunoglobulin G₁. It is possible to fuse the entire heavy chain constant region to the sequence containing the receptor binding site(s). However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site (which defines IgG Fc chemically; residue 216, taking the first residue of heavy chain constant region to be 114 [Kobet et al., Supra], or analogous sites of other immunoglobulins) is used in the fusion. In a particularly preferred embodiment, the amino acid sequence containing the receptor binding site(s) is fused to the hinge region and C_H2 and C_H3 or C_H1, hinge, C_H2 and C_H3 domains of an IgG₁, IgG₂ or IgG₃ heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

HGF variant-immunoglobulin chimeras may, for example, be used in protein A purification, immunohistochemistry, and immunoprecipitation techniques in place of anti-HGF antibodies, and can facilitate screening of inhibitors of HGF-HGF receptor interactions.

Therapeutically, they are expected to confer advantages such as longer half-life as compared to the corresponding HGF variant molecule.

IV. Therapeutic Compositions

5 The HGF variants with enhanced receptor binding affinity can be used to block the binding of wild-type HGF to its receptor. This would permit the treatment of pathologic conditions associated with the activation of an HGF receptor, such as malignancies associated with chronic HGF receptor activation.

10 The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the HGF product is combined in admixture with a pharmaceutically acceptable carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences,
15 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. These compositions will typically contain an effective amount of the HGF variant, for example, from on the order of about 0.5 to about 10 mg/ml, together with a suitable amount of carrier to prepare pharmaceutically acceptable compositions suitable for effective
20 administration to the patient. The variants may be administered parenterally or by other methods that ensure its delivery to the bloodstream in an effective form.

 Compositions particularly well suited for the clinical administration of the HGF variants used to practice this invention
25 include sterile aqueous solutions or sterile hydratable powders such as lyophilized protein. Typically, an appropriate amount of a pharmaceutically acceptable salt is also used in the formulation to render the formulation isotonic.

 Dosages and desired drug concentrations of pharmaceutical
30 compositions of this invention may vary depending on the particular use envisioned. A typical effective dose in rat experiments is about 250 µg/kg administered as an intravenous bolus injection. Interspecies scaling of dosages can be performed in a manner known in the art, e.g. as disclosed in Mordenti et al., Pharmaceut. Res. 8,
35 1351 (1991) and in the references cited therein.

 The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention.

V. Examples

A series of recombinant huHGF (rhuHGF) variants were produced to determine the structural and functional importance of the cleavage of the prohormone to the α/β dimer and of the Kringle and protease-like domains. Mutations were introduced into the huHGF cDNA in a CMV based expression plasmid and conditioned media from stable populations of human 293 cells expressing each variant were assayed by Western blotting to monitor the size and expression level of the HGF variants.

The concentration of each huHGF derivative was confirmed with two types of sandwich ELISA assays. The differences in expression levels found in ELISA correlated with those observed on Western blots. For most variants, the level of expression was in the range of 1-5 mg/mL. For variants with expression levels below 0.6 mg/mL, the conditioned media was concentrated.

The mitogenic activity on liver cells in primary culture and ability to bind to the HGF receptor was then determined. The extracellular domain of the HGF receptor was fused to the constant region (Fc) of an human IgG and binding was performed in solution.

The construction of the rhuHGF variants, the assay methods and the analysis of the results obtained with the various mutants are described in the following examples.

EXAMPLE 1Recombinant Production of the huHGF Variants

A. Site-directed mutagenesis

Plasmid DNA isolation, polyacrylamide and agarose gel electrophoresis were performed as disclosed in Sambrook *et al.*, *supra*.

Mammalian expression plasmid pRK 5.1 with a CMV promotor (Genentech, Inc.) was used for mutagenesis of huHGF allowing secretion of the HGF variants in the culture medium and directly assayed for biological activity and binding. This expression vector is a derivative of pRK5, the construction of which is disclosed in EP 307,247 published 15 March 1989. The nucleotide sequence encoding this the pRK 5.1 vector is shown in Figure 5 (SEQ. ID. NO: 1).

The huHGF cDNA used corresponds to the 728 amino acid form as published earlier (Miyazawa *et al.*, 1989, *supra*).

Mutagenesis was performed according to the method of Kunkel using the commercially available dut- ung- strain of *E. coli* [Kunkel *et*

al., Method. Enzymol. 154, 367-382 (1987)]. Synthetic oligonucleotides used for in vitro mutagenesis and sequencing primers were prepared using the Applied Biosystem 380A DNA synthesizer as described [Matteucci et al., J. Am. Chem. Soc. 103, 3185-3191 (1981)].

5 For generation of the desired mutants, oligonucleotides of sequences coding for the desired amino acid substitutions were synthesized and used as primers. The oligonucleotides were annealed to single-stranded pRK51-huHSA that had been prepared by standard procedures [Viera et al., Method. Enzymol. 142, 3 (1987)].

10 A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), was combined with a modified thio-deoxyribonucleosine called dCTP(aS) provided in the kit by the manufacturer, and added to the single stranded pRK 5.1-huHGF to which was annealed the oligonucleotide.

15 Upon addition of DNA polymerase to this mixture, a strand of DNA identical to pRK 5.1-huHGF except for the mutated bases was generated. In addition, this new strand of DNA contained dCTP(aS) instead of dCTP, which served to protect from restriction endonuclease digestion. After the template strand of the double-stranded heteroduplex was
20 nicked with an appropriate restriction enzyme, the template strand was digested with ExoIII nuclease past the region that contained the mutagenic oligomer. The reaction was then stopped to leave a molecule that was only partly single-stranded. A complete double-stranded DNA homoduplex molecule was then formed by DNA polymerase in the presence
25 of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase.

The following oligonucleotides were prepared to use as primers to generate pRK 5.1-huHGF variant molecules:

R494E huHGF: TTGGAATCCCATTTACAACCTCGAGTTGTTTCGTTTTGGCACAAGAT

(SEQ. ID. NO: 2)

30 R494D huHGF: GAATCCCATTTACGACGTCCAATTGTTTCG (SEQ. ID. NO: 3)

R494A huHGF: CCCATTTACAACCTGCCAATTGTTTCG (SEQ. ID. NO: 4)

Q534H huHGF: AGAAGGGAAACAGTGTCTGCA (SEQ. ID. NO: 5)

Y673S huHGF: AGTGGGCCACCAGAATCCCCCT (SEQ. ID. NO: 6)

V692S huHGF: TCCACGACCAGGAGAAATGACAC (SEQ. ID. NO: 7)

35 ΔK1 huHGF: GCATTCAACTTCTGAGTTTCTAATGTAGTC (SEQ. ID. NO: 8)

ΔK2 huHGF: CATAGTATTGTCAGCTTCAACTTCTGAACA (SEQ. ID. NO: 9)

ΔK3 huHGF: TCCATGTGACATATCTTCAGTTGTTTCCAA (SEQ. ID. NO: 10)

ΔK4 huHGF: TGTGGTATCACCTTCATCTTGTCCATGTGA (SEQ. ID. NO: 11)

N-303 huHGF: ACCTTGGATGCATTAAGTTGTTTC (SEQ. ID. NO:12)

N-384 huHGF: TTGTCCATGTGATTAATCACAGT (SEQ. ID. NO:13)

α -chain: GTTCGTGTTGGGATCCCATTTACCTATCGCAATTG (SEQ. ID. NO:14)

5 The Y673S, V692S huHGF variant was obtained from wild-type huHGF as a template, using both oligonucleotides used for generating the two mutations.

10 The mutant huHGF constructs generated using the protocol above were transformed in *E. coli* host strain MM294tonA using the standard calcium chloride procedure (Sambrook *et al.*, *supra*) for preparation and transformation of competent cells. MM294tonA (which is resistant to T1 phage) was prepared by the insertion and subsequent imprecise excision of a Tn10 transposon into the *tonA* gene. This gene was then inserted, using transposon insertion mutagenesis [Kleckner *et al.*, *J. Mol. Biol.* 116, 125-159 (1977)], into *E. coli* host MM294 (ATCC 15 31,446).

The DNA extract from individual colonies of bacterial transformants using the standard miniprep procedure of Sambrook *et al.*, *supra*. The plasmids were further purified by passage over a Sephacryl CL6B spin column, and then analyzed by sequencing and by 20 restriction endonuclease digestion and agarose gel electrophoresis.

B. Transfection of Human Embryonic Kidney 293 Cells

Plasmids with the correct sequence were used to transfect human fetal kidney 293 cells by the calcium phosphate method. 293 cells were growth to 70% confluence in 6-well plates. 2.5 μ g of huHGF plasmid 25 DNA variant was dissolved in 150 μ l of 1 mM Tris-HCl, 0.1 mM EDTA, 0.227 M CaCl_2 . Added to this (dropwise while vortexing) was 150 μ l of 50 mM HEPES buffer (pH 7.35), 280 mM NaCl, 1.5 mM NaPO_4 , and the precipitate was allowed to form for ten minutes at 25 °C. The suspended precipitate was then added to the cells in the individual 30 wells in a 6-well plate. The cell monolayers were incubated for 4 hours in the presence of the DNA precipitate, washed once with PBS, and cultured in serum-free medium for 72h. When stable populations were made, the HGF cDNA was subcloned in an episomal CMV driven expression plasmid pCisEBON (G. Cachianes, C. Ho, R. Weber, S. Williams, D. Goeddel, and D. Lueng, in preparation). pCisEBON is a 35 pRK5 derivative; its underlying nucleotide sequence is shown in Figure 6 (SEQ. ID. NO: 15). The populations were directly selected in Neomycin selective medium.

EXAMPLE 2Assay Methods

In view of the pleiotropic activities of HGF, a molecule with a structure unlike any other known growth factor, it is important to understand the molecular interaction of this factor with its receptor. The huHGF variants produced as described in Example 1 were analyzed for their ability to induce DNA synthesis of hepatocytes in primary culture and to compete for binding to a soluble form of the huHGF receptor.

10 A. Protein quantification of wild-type huHGF and huHGF variants.

A specific two-site huHGF sandwich ELISA using two monoclonal antibodies was used to quantify wild-type recombinant huHGF (WT rhuHGF), single chain and protease substitution variants. Microtiter plates (Maxisorb, Nunc) were coated with 10 mg/ml of a monoclonal anti-rhuHGF antibody A 3.1.2 (IgG2a phenotype, affinity: 3.2×10^{-8} mol) in 50 mM Carbonate buffer, pH 9.6, overnight at 4°C. After blocking plates with 0.5 % BSA (Sigma), 0.01 % thimerosal in PBS, pH 7.4, and subsequent washes, duplicate serial dilutions of HGF samples were prepared and in parallel a CHO-expressed rhuHGF (40-0.1 ng/mL) was used as a standard. Fifty microliters of these dilutions were simultaneously incubated with 50 mL of a 1:1500 diluted horseradish peroxidase conjugated monoclonal anti-rhuHGF antibody B 4.3 (IgG1 phenotype, affinity: 1.3×10^{-8} mol) for 2 h at RT. The substrate was prepared by adding 0.04 % o-phenylenediamine-dihydrochloride (Sigma) and 0.012 % (v/v) hydrogen-peroxide (Sigma) to PBS and 100 ml were added to the washed plates for 15 minutes at RT. The reaction was stopped by adding 50 mL of 2.25 M sulfuric acid to each well. The absorbance at 490 nm, with the absorbance at 405 nm subtracted as background, was determined on a microtiter plate reader (Vmax, Molecular Devices, Menlo Park, CA). The data was reduced using a four-parameter curve-fitting program developed at Genentech, Inc.

An HGF polyclonal sandwich ELISA was used to quantify all kringle deletion and C-terminal truncation variants. Briefly, microtiter plates (Nunc) were coated with 5 mg/mL guinea pig polyclonal (anti CHO-expressed rhuHGF) IgG antibody preparation (Genentech, Inc.) as described above. This antibody recognizes rhuHGF as well as HGF truncated forms when compared to visual inspection of Western blots, making it ideal for monitoring HGF variants. Plates were blocked and

duplicate serial dilutions of 293 cell supernatants (1:103-6.106) were added and incubated over night at 4°C. Purified CHO-expressed rhuHGF (100-0.78 ng/mL) was used as a standard and incubated in parallel. Plates were washed and incubated with a 1:500 dilution of the same polyclonal antibody (approx. 400 ng/mL) but in this case horseradish peroxidase conjugated for detection of the variants (see above). Western blotting was performed to determine the size of the expressed HGF variants. For this, SDS-polyacrylamide gel electrophoresis and Western blotting were performed using standard methods with the polyclonal IgG antibody preparation (500 ng/mL). A chemiluminescent detection method (Amersham) and a goat anti-guinea pig IgG-horseradish peroxidase conjugate (1:5000) were used for development of the blot as described by the manufacturer.

B. Soluble HGF receptor binding assay.

Previous studies on HGF binding to hepatocytes have shown that huHGF could bind to its cell surface receptor with high affinity (K_d 24-32 pM; Higuchi and Nakamura, Biochem. Biophys. Res. Comm. 174, 831-838 (1991)). We preferred to examine HGF binding using a soluble form of the receptor because of the nonspecific binding of HGF to cell surface heparin sulfate proteoglycans [Naldini et al., EMBO J. 10, 2867-2878 (1991)].

Cell supernatants (concentrated on Amicon filters if concentration was below 600 ng/mL) were tested for their ability to block in solution the binding of CHO-expressed 125 I rhuHGF (2-5 x 10³ Ci/mole, kindly provided by T. Zioncheck, Genentech, Inc.) to the extracellular domain of the human HGF receptor (huHGFr) fused to the Fc constant region of an human IgG, expressed and secreted from 293 cells.

1.. Construction of huHGFr-IgG chimeras.

A full length cDNA clone encoding the huHGFr was constructed by joining partial cDNAs isolated from cDNA libraries and from PCR amplification. Coding sequences for amino acids 1-270 were isolated from a human placental cDNA library (provided by T. Mason, Genentech) screened with a 50 mer oligonucleotide (5'-ATGAAGGCCCCGCTGTGCTTGACCTGGCATCCTCGTGCTCCTGTTTACC-3') (SEQ. ID. NO: 16). Sequences encoding amino acids 809-1390 were isolated from a human liver library (Stragagen) screened with the oligonucleotide probe

(5'-CACTAGTTAGGATGGGGGACATGTCTGTCAGAGGATACTGCACTTGTCTGGCATGAA CCGT-3').
(SEQ. ID. NO: 17)

Conditions for plating libraries, and for hybridization and washing filters were as described [Godowski *et al.*, Proc. Natl. Acad. Sci. USA **86**, 8083-8087 (1989)]. PCR was used to isolate a cDNA clone containing residues 271-808 of the HGFr (c-met) from A549 cells. Ten μ g of total RNA was used for reverse transcription using a primer specific to the HGFr (5'-TAGTACTAGCACTATGATGTCT -3') (SEQ. ID. NO: 18) in a 100 μ l reaction using Moloney murine leukemia virus reverse transcriptase and buffers supplied by Bethesda Research Laboratories. One-tenth of this reaction mixture was used for PCR amplification. The PCR reaction was performed in a volume of 100 μ l containing 10 μ l of the reverse transcriptase reaction, 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM (NH₄)SO₄, 6 mM MgSO₄, 0.1% Triton X-100, 1 U of Vent DNA polymerase (New England Biolabs) and 50 pmol each of the forward primer (5'-TTTACTTCTTGACGGTCCAAAG-3' (SEQ. ID. NO: 19) and the reverse primer (5'-CAGGGGGAGTTGCAGATTCAGCTGT-3') (SEQ. ID. NO: 20). After thirty cycles of denaturation (95°C, 1 min), annealing (55°C, 45 secs) and extension (72°C, 2 min), the PCR product were recovered from low-melting temperature agarose gels. The full-length HGFr cDNA was subcloned into vector pRK7 (see WO 90/02798, published 22 March 1990) and double-stranded DNA sequencing was performed by the dideoxynucleotide method.

The coding sequence of the extracellular domain of the huHGFr was fused to those of the human IgG1 heavy chain in a two-step process. PCR was used to generate a fragment with a unique BstEII site 3' to the coding sequences of the HGFr amino acid 929. The 5' primer (located in the vector upstream of the HGFr coding sequences) and the 3' primer (5'-AGTTTGTGCGGTGACCTGATCATTCTGATCTGGTTGAACTATTAC-3') (SEQ. ID. NO: 21) were used in a 100 μ l reaction as described above except that the extension time at 72°C was 3 minutes, and 40 ng of the full length HGFr expression vector was used as template. Following amplification, the PCR product was joined to the human IgG- γ 1 heavy chain cDNA through a unique BstEII site in that construct [Bennett *et al.*, J. Biol. Chem. **266**, 23060-23067 (1991)]. The resulting construct contained the coding sequences of amino acids 1-929 of the huHGFr fused via the BstEII site (adding the coding sequences for amino acids V and T) to the coding sequences of amino acids 216-443 of the human

IgG- γ 1 heavy chain. Sequencing of the construct was carried out as described above.

2. Binding assay.

The binding assay was performed in breakable microtiter plates (Nunc) coated o/n at 4°C with 1 mg/mL of rabbit-anti-human IgG Fc specific antibody (Jackson ImmunoResearch) and plates were carefully washed with PBS containing 0.05% Tween 20 (Biorad). After blocking with PBS containing 0.1% BSA, in this same buffer, 50pM of ¹²⁵I-rhuHGF in 25 mL per well were added. To each well 50 mL of serial dilutions (1:25-1:6000) of cell supernatants, purified CHO-expressed rhuHGF (25,000-0.064 pM) or medium were added in duplicates. Subsequently, 25 mL of 50 pM of HGF receptor:IgG fusion protein were added and the plates were incubated with gentle shaking. After 4 hours, when equilibrium was reached, plates were washed and wells were individually counted in a gamma-counter. The amount of nonspecifically bound radioactivity was estimated by incubating HGF receptor:IgG with a 500-fold excess of unlabelled rhuHGF. The dissociation constant (Kd) of each analogue was calculated at the IC₅₀ from fitted inhibition curves essentially as described (DeBlasi et al., 1989 [?]) using the huHGF concentration determined by ELISA.

C. Biological assay.

The biological activity of WT huHGF and variants was measured by their abilities to induce DNA synthesis of rat hepatocytes in primary culture. Hepatocytes were isolated according to published perfusion techniques with minor modifications [Garrison and Haynes, J. Biol. Chem. **150**, 2269-277 (1975)]. Briefly, the livers of female Sprague Dawley rats (160-180g) were perfused through the portal vein with 100 mL of Ca⁺⁺ free Hepes buffered saline containing 0.02% Collagenase type IV (Sigma). After 20 minutes the liver was removed, placed in buffer, gently stirred to separate hepatocytes from connective tissue and blood vessels, and filtered through nylon mesh. Cells were then washed by centrifugation, resuspended at 1x10⁵ cells/mL in Williams Media E (Gibco) containing Penicillin (100 U/ml), Streptomycin (100 mg/mL), L-Glutamine (2mM), trace elements (0.01%), transferrin (10 mg/mL) and Aprotinin (1 mg/mL). Hepatocytes were incubated in 96-well microtiter plates (Falcon) in the presence of duplicate serial dilutions of either purified CHO-expressed rhuHGF (1-0.031 mg/mL), 293 supernatants (1:4-1:256) or medium. After 48 hours incubation at

37°C, 0.5 mCi 3H-TdR (15 Ci/mmol, Amersham) was added to each well and incubated for an additional 16 hours. Cells were harvested on filter papers, which were washed, dried and counted in a Beckman counter after addition of scintillation liquid. For each huHGF variant, the specific activity (SA) expressed in units/mg was calculated at half-maximal proliferation (defined as 1 unit/mL) using the HGF concentration obtained in ELISA.

D. Induction of tyrosine phosphorylations on A549 cells.

Human lung carcinoma cells (A549) monolayers were cultured in RPMI 1640 medium containing 10% fetal bovine serum and maintained at 37°C in a humidified atmosphere with 5% CO₂. Serum-starved cells were incubated without or with 200 ng/mL rhuHGF for 5 minutes at 37°C and extracted with lysis buffer containing 50 mM Hepes, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10 % Glycerol, 1 % Triton X-100 and a cocktail of protease inhibitors. The lysates were immunoprecipitated with anti-Met COOH antibodies and blotted with anti-phosphotyrosine antibodies (see Western blotting above).

EXAMPLE 3

Analysis of Cleavage Site Mutants

The cleavage site of proteases commonly contains a basic residue at position P1 and two hydrophobic amino acid residues in positions P'1 and P'2, which follow the cleaved peptide bond. The proposed cleavage site of huHGF (P1 R494, P'1 V495, P'2 V496) fits this consensus. We chose to try to block cleavage of huHGF by replacing the P1 R494 with either D, E, or A. The major form of WT rhuHGF expressed in these cells is cleaved into two-chain material as judged by the presence of the α -chain with an apparent molecular mass of 69 kDa (Fig. 2). Each of these mutations appeared to block processing of rhuHGF because under reducing conditions these variants migrated as a single band at 94 kDa, the predicted size of single-chain HGF. These variants totally lacked the ability to induce the proliferation of hepatocytes in primary culture (Fig. 3A). However, when these variants were analyzed for their ability to compete with WT rhuHGF for binding to the HGF receptor:IgG fusion protein, their inhibition curves were roughly similar to that of WT rhuHGF (Fig. 3B). The K_d determined from these curves showed that WT rhuHGF binds to the fusion protein with high affinity (50-70pM) whereas all single chain variants showed

approximately a 2- to 10-fold higher Kd (100-500pM) compared to WT rhuHGF. Results from at least three independent assays are summarized in Table I as residual hepatocyte proliferative activity and receptor binding capacity compared to WT rhuHGF.

5 Our binding studies showed that WT rhuHGF bound to the soluble receptor fusion protein with a single class of high affinity binding sites (50-70 pM), similar to those found on hepatocytes by Higushi and Nakamura (1991). However, binding of HGF on cells may slightly be different since the soluble receptor is actually a dimer held together
10 by the disulfide bridge of the hinge in the Fc portion of the IgGA.

Direct comparison of specific activity (SA) versus Kd ratios of all single chain variants showed they were inactive at the highest concentration tested (SA< 3%) while receptor binding affinities were only decreased by a factor of 2-3.

15 These results argue strongly that cleavage of HGF into the two-chain form is required for mitogenic activity, i.e. that single-chain HGF is a promitogen and that the uncleaved form of HGF binds to the HGF receptor, albeit with a reduced affinity.

20 The major form of HGF isolated from placenta [Hernandez et al., (1992) J. Cell Physiol., in press] or expressed in transfected COS cells [Rubin et al., Proc. Natl. Acad. Sci. USA 88, 415-419 (1991)] is in single-chain form. When tested in mitogenic assays, this single-chain form of HGF is found to be biologically active. Taken together with our data, this suggests that this single-chain HGF is activated
25 to the two-chain form during the mitogenic assay.

A second observation is that single-chain variants retain substantial capacity to bind to the HGF receptor, as suggested by our competition binding assays. This raises the interesting possibility that single-chain HGF may be bound to cell-surface HGF receptor in
30 vivo in an inactive state and can subsequently be cleaved to the active double-chain form by the appropriate protease.

EXAMPLE 4

The Effects of Protease Domain Mutations

35 To elucidate the functional importance of the protease domain of HGF, several single, double and triple mutations were made in order to reconstitute a potential serine-protease active site. The construction of these variants is described in Example 1.

We replaced HGF residues Q534 with H, Y673 with S, or V692 with S as either single, double or triple mutations. The analysis of their effects on mitogenic activity and receptor binding showed that the single mutation Q534H did not significantly alter either SA (5.2×10^4 Units/mg) or Kd (60 pM) when compared to wt rhuHGF (respectively 3.3×10^4 Units/mg and 70 pM) whereas Y673S and V692S exhibited SA reduced approximately 5- and 10-fold, respectively. In fact, these two variants never reached the maximum plateau seen with WT rhuHGF (approximately 50 % of wt rhuHGF plateau). Interestingly, these variants showed a Kd similar to WT rhuHGF. All other double and triple variants also retained the ability to bind the HGF receptor but they clearly showed a reduced SA (Table I). The residual SA of the double variants Q534H,Y673S and Y673S,V692S and of the triple variant Q534H,Y673S,V692S were less than 3 % compared to WT rhuHGF. However, the Kd of these variants was not significantly different from WT rhuHGF (Table I). These variants indicate that mutations within the β -chain of HGF block mitogenic activity but they are still able to bind to the HGF receptor. Thus, it appears that these mutants are defective in an activity subsequent to receptor binding.

These results show that although the β -chain is not required for receptor binding, certain residues (e.g. Y673 and V692) are critical for the structure and/or activity of HGF. Substitution of the nonpolar residue V692 with the polar residue S might have caused a structural transition if new hydrogen bonds to the active site residue D594, as found in serine-proteases, have been introduced. Substitution of Y673 with the smaller residue S might also introduce some local structural modifications. On the other hand, replacement of the polar residue Q534 by another polar residue H of similar size would not likely cause a drastic difference in the HGF conformation as this residue should be exposed; indeed the Q534H variant was similar to rhuHGF (Table I).

EXAMPLE 5

The Effect of C-terminal and Kringle Deletions

In order to ascertain whether the α -chain is required for HGF binding or activity, C-terminal truncations were made as described in Example 1, resulting in variants containing either the α -chain alone,

or variants truncated after the third (N-384) or second (N-303) Kringles.

A number of C-terminal truncations of HGF were made by deleting either the β -chain or the β -chain in addition to a progressive number of kringles as depicted in Fig. 1. One variant (N-207) corresponding to the N-terminal domain with the first Kringle did not express the protein to levels detectable either by Western blotting or ELISA using a polyclonal antibody preparation and thus was not investigated further. Expression of the variants containing the first two Kringles (N-303), three Kringles (N-384) or the complete α -chain of HGF was as low as 250-600 ng/mL. A summary of the residual SA and Kd compared to WT rhuHGF of these variants is presented in Table I. At the concentration tested no activity above background levels was observed indicating that these variants lost their biological activity. However, binding competition showed that variants N-303, N-384 or the α -chain still retained substantial binding capacity (up to 23 % compared to WT rhuHGF binding). Thus, the N-terminal 272 residues of HGF (the mature form of variant N-303) are sufficient for high affinity binding to the HGF receptor. Results from deleting each kringle domain are shown in Table 1. Deletion of the first Kringle (variant AK1) of HGF affected biological activity most, showing at least a 100-fold reduction (SA < 0.2% of wt rhuHGF). Similarly, binding of this variant was also affected as it failed to compete for binding with wt rhuHGF up to 2 mg/mL. Deletion of all other Kringles (variants AK2, AK3 or AK4) also induces severely reduced mitogenic activity (Table I). However, the Kds of these deletion variants remained close to that observed with wt rhuHGF.

These data show that Kringles K3 and K4 are not required for receptor binding. Our data support the previous observations by Miyazawa *et al.*, 1991 *supra* and Chan *et al.*, 1991 *supra*, in the sense that variant N-303, which in amino acid sequence is very similar to HGF/NK2, retains the ability to compete efficiently for binding to the HGF receptor (Kd ~ 280 pM). Furthermore, the observations that N-303 is sufficient to bind to the receptor and that the second Kringle is not required for binding the HGF receptor (in the context of the remainder of the molecule) suggest that the receptor binding domain is contained within the finger and first Kringle of huHGF. Unfortunately, we have not been able to detect expression of this

variant using our polyclonal antisera suggesting that variant N-207 (deletion after the first kringle) was not expressed in 293 cells.

EXAMPLE 6

5 Induction of Tyrosine-Phosphorylation of the huHGF Receptor

We determined if variants R494E or Y673S,V692S, which bind the HGF receptor in vitro but are defective for mitogenic activity, could stimulate tyrosine-phosphorylation of the HGF receptor in A549 cells. Serum starved cells were treated with purified WT rhuHGF or variants
10 and immunoprecipitates of the HGF receptor were blotted and probed with phosphotyrosine antibodies. Stimulation with wt rhuHGF led to the phosphorylation on tyrosine of the 145 kDa β -subunit of the HGF receptor (Fig. 4). Both variants exhibited a reduced ability to induce phosphorylation of the HGF receptor.

15 Stimulation of tyrosine phosphorylation on the HGF receptor β -subunit by HGF was previously reported [Bottaro et al., Science 251, 802-804 (1991), Naldini et al., 1991 supra]. The present data show that variants R494E and Y673S,V692S can bind the soluble HGF receptor: IgG protein in vitro but are not efficient in stimulating tyrosine-
20 phosphorylation in A549 cells. One interpretation of this result is that these variants are capable of binding the HGF receptor on A549 cells, but are defective in a function required to induce efficient phosphorylation, e.g. receptor dimerization. It has been shown for other receptor proteins with an intrinsic tyrosine kinase such as the
25 epithelial and platelet-derived growth factor that receptor-receptor interactions or dimerization is required for activation of kinase function [see for review Ulrich and Schlessinger, Cell 61 203-212 (1990)]. Alternatively, these variants may not be able to bind the cell-surface associated HGF receptor.

30 The unique structure of HGF suggests that there may be multiple events that regulate the biological activity of this molecule. An early stage of regulation may be the cleavage step to generate the biologically active two-chain form. Interestingly, cleavage may not simply regulate receptor binding but rather control a subsequent event
35 required for activating the HGF receptor. Our data also suggest that the β -chain, while not absolutely required for receptor binding contributes to a receptor activation step. These variants may be useful in dissecting the signalling events at the HGF receptor.

EXAMPLE 7Hairpin Domain and Kringle 1 Domain Variants

The huHGF variants listed in Tables 2 and 3 were generated, and their specific activities (SA) and Kd ratios were determined
5 essentially as described in the foregoing examples.

Although the foregoing refers to particular preferred
embodiments, it will be understood that the present invention is not
so limited. It will occur to those ordinarily skilled in the art that
10 various modifications may be made to the disclosed embodiments without
diverting from the overall concept of the invention. All such
modifications are intended to be within the scope of the present
invention.

15

Table 1

	Variants (var)	SA var/SA wt	Kdwt/Kdvar
		+/- S.D.	+/- S.D.
	Single-chain		
5	R494A	<0.03	0.32 +/- 0.18
	R494D	<0.03	0.51 +/- 0.21
	R494E	<0.02	0.31 +/- 0.13
	Protease		
10	Q534H	1.19 +/- 0.44	1.48 +/- 0.85
	Y673S	0.27 +/- 0.07*	1.35 +/- 0.72
	V692S	0.08 +/- 0.04	1.02 +/- 0.13
	Q534H,Y673S	<0.03	2.24 +/- 1.11
	Y673S,V692S	<0.02	1.76 +/- 0.63
	Q534H, Y673S, V692S	<0.02	1.91 +/- 1.28
	C-terminal truncation		
15	N-303	<0.05	0.23 +/- 0.03
	N-384	<0.05	0.25 +/- 0.02
	α -chain	<0.04	0.25 +/- 0.03
	Kringle deletion		
20	Δ K1	<0.002	<0.03
	Δ K2	<0.05	0.41 +/- 0.18
	Δ K3	<0.03	0.56 +/- 0.36
	Δ K4	<0.07	0.86 +/- 0.46

25 * means that the mitogenic activity of the variant did not reach the same absolute level as wild-type huHGF.

Table 2

HGF variant	n	SA mut/wt +/-SD	n	Kdwt/mut
wt rhuHGF	3	1	3	1
K137A	3	1.12 +/- 0.10	3	0.98 +/- 0.04
K144A, K148A	3	0.93 +/- 0.16	3	1.03 +/- 0.08
E159A	3	< 0.02	3	< 0.03
S161A	3	0.15 +/- 0.03*	3	0.06 +/- 0.04
F162A, L163A, S165A, S166A	3	< 0.02	3	0.05 +/- 0.02
F162A	3	0.04 +/- 0.01*	3	0.05 +/- 0.01
L163A, S165A, S166A	3	0.14 +/- 0.08	3	1.10 +/- 0.04
Y167A	3	1.22 +/- 0.22*	3	0.92 +/- 0.06
Y167F	3	0.38 +/- 0.03*	3	1.02 +/- 0.02
delta5-Y167A	3	< 0.02	3	< 0.02
delta5-Y167F	3	0.11 +/- 0.02	3	1.01 +/- 0.13
R168A	3	0.83 +/- 0.07	3	0.98 +/- 0.04
Q173A, E174A, N175A	3	0.33 +/- 0.07*	3	0.21 +/- 0.03
Q173A	3	0.13 +/- 0.03*	3	0.15 +/- 0.05
E174A	3	0.84 +/- 0.11	3	0.99 +/- 0.02
R181A, E183A, E184A	3	0.92 +/- 0.08	3	0.95 +/- 0.04
N193A, E195A, R197A	3	< 0.02	3	< 0.04
N193A	3	0.45 +/- 0.12	3	0.62 +/- 0.20
R195A	3	0.10 +/- 0.06	3	0.17 +/- 0.05
R197A	3	< 0.01	3	< 0.03
Y198A	3	0.88 +/- 0.06	2	0.77

* means that the mitogenic activity of the variant did not reach the same absolute level as wild-type huHGF.

Table 3
Variants with alterations near or within the Hairpin Domain

HGF variant	n	SA +/- mut/wt	n	Kd wt/mut
wt rhuHGF	3	1	3	1
delta-hairpin	3	< 0.01	3	< 0.02
K34A, R35A, R36A	3	0.71 +/- 0.33	3	0.95 +/- 0.03
K52A, D54A	3	0.32 +/- 0.03*	3	0.95 +/- 0.03
K52A	3	0.19 +/- 0.03	3	0.89 +/- 0.05
D54A	3	0.13 +/- 0.01	3	0.79 +/- 0.10
K58A, K60A, K62A, K63A, N65A	3	0.96 +/- 0.12	3	0.99 +/- 0.07
N72A, R76A, N77A, K78A	3	0.04 +/- 0.07	3	0.83 +/- 0.11
K109A, K110A, E111A	3	0.98 +/- 0.04	3	0.78 +/- 0.02
H114A, E115A, D117A	3	< 0.02	3	< 0.04
H114A	3	0.16 +/- 0.04*	2	0.59
E115A	3	0.20 +/- 0.02*	2	0.47
D117A	3	0.02*	2	0.14
R126A, N127A	3	0.33 +/- 0.14	3	0.87 +/- 0.12

* means that the mitogenic activity of the variant did not reach the same absolute level as wild-type huHGF.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Genentech, Inc., Godowski, Paul J., Lokker, Natalie
A., Mark, Melanie R.
- (ii) TITLE OF INVENTION: HEPATOCYTE GROWTH FACTOR VARIANTS
- 10 (iii) NUMBER OF SEQUENCES: 21
- (iv) CORRESPONDENCE ADDRESS:
- 15 (A) ADDRESSEE: Genentech, Inc.
(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080
- 20 (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: patin (Genentech)
- 25 (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- 30 (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 07/884811
(B) FILING DATE: 18-MAY-92
- 35 (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 07/885971
(B) FILING DATE: 18-MAY-92
- (viii) ATTORNEY/AGENT INFORMATION:
- 40 (A) NAME: Dreger, Ginger R.
(B) REGISTRATION NUMBER: 33,055
(C) REFERENCE/DOCKET NUMBER: 755,779P1
- (ix) TELECOMMUNICATION INFORMATION:
- 45 (A) TELEPHONE: 415/225-3216
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168
- (2) INFORMATION FOR SEQ ID NO:1:
- 50 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 4732 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
55 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

5 TTCGAGCTCG CCCGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT 50

TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC GTTACATAAC 100

10 TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG 150

ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA 200

15 TTGACGTCAA TGGGTGGAGT ATTTACGGTA AACTGCCCAC TTGGCAGTAC 250

ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT CAATGACGGT 300

20 AAATGGCCCG CCTGGCATTG TGCCAGTAC ATGACCTTAT GGGACTTTCC 350

25 TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC 400

GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA 450

30 TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA 500

AAATCAACGG GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC 550

35 AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG CAGAGCTCGT 600

40 TTAGTGAACC GTCAGATCGC CTGGAGACGC CATCCACGCT GTTTTGACCT 650

CCATAGAAGA CACCGGGACC GATCCAGCCT CCGCGGCCGG GAACGGTGCA 700

45 TTGGAACGCG GATTCCCCGT GCCAAGAGTG ACGTAAGTAC CGCCTATAGA 750

GTCTATAGGC CCACCCCCTT GGCTTCGTGA GAACGCGGCT ACAATTAATA 800

50 CATAACCTTA TGTATCATAC ACATACGATT TAGGTGACAC TATAGAATAA 850

55 CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACCTGC 900

ACCTCGGTTT TATCGATTGA ATTCCCCGGG GATCCTCTAG AGTCGACCTG 950

5 CAGAAGCTTG CCTCGAGGCA AGCTTGCCCG CCATGGCCCA ACTTGTTTAT 1000

TGCAGCTTAT AATGGTTACA AATAAAGCAA TAGCATCACA AATTTACAA 1050

10 ATAAAGCATT TTTTTCCTG CATTCTAGTT GTGGTTTGTC CAAACTCATC 1100

AATGTATCTT ATCATGTCTG GATCGATCGG GAATTAATTC GGCGCAGCAC 1150

15 CATGGCCTGA AATAACCTCT GAAAGAGGAA CTTGGTTAGG TACCTTCTGA 1200

GGCGGAAAGA ACCAGCTGTG GAATGTGTGT CAGTTAGGGT GTGGAAAGTC 1250

20 CCCAGGCTCC CCAGCAGGCA GAAGTATGCA AAGCATGCAT CTCAATTAGT 1300

CAGCAACCAG GTGTGGAAAG TCCCCAGGCT CCCAGCAGG CAGAAGTATG 1350

CAAAGCATGC ATCTCAATTA GTCAGCAACC ATAGTCCCGC CCCTAACTCC 1400

30 GCCCATCCCG CCCCTAATC CGCCCAGTTC CGCCCATTTCT CCGCCCCATG 1450

GCTGACTAAT TTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT 1500

35 GAGCTATTCC AGAAGTAGTG AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG 1550

CAAAAAGCTG TTAACAGCTT GGCCTGGCC GTCGTTTTAC AACGTCGTGA 1600

CTGGGAAAAC CCTGGCGTTA CCCAACTTAA TCGCCTTGCA GCACATCCCC 1650

45 CCTTCGCCAG CTGGCGTAAT AGCGAAGAGG CCCGCACCGA TCGCCCTTCC 1700

CAACAGTTGC GTAGCCTGAA TGGCGAATGG CGCCTGATGC GGTATTTTCT 1750

50 CCTTACGCAT CTGTGCGGTA TTTCACACCG CATACTCAA AGCAACCATA 1800

55 GTACGCGCCC TGTAGCGGCG CATTAGCGC GGCGGGTGTG GTGGTTACGC 1850

GCAGCGTGAC CGCTACACTT GCCAGCGCCC TAGCGCCCGC TCCTTTCGCT 1900

5 TTCTTCCCTT CCTTCTCGC CACGTTCCGC GGCTTTCCCC GTCAAGCTCT 1950

AAATCGGGGG CTCCCTTTAG GGTTCGATT TAGTGCTTTA CGGCACCTCG 2000

10 ACCCCAAAAA ACTTGATTG GGTGATGGT CACGTAGTGG GCCATCGCCC 2050

TGATAGACGG TTTTTCGCCC TTTGACGTTG GAGTCCACGT TCTTTAATAG 2100

15 TGGACTCTTG TTCCAAACTG GAACAACACT CAACCCTATC TCGGGCTATT 2150

CTTTTGATTT ATAAGGGATT TTGCCGATTT CGGCCTATTG GTTAAAAAAT 2200

20 GAGCTGATTT AACAAAAATT TAACGCGAAT TTTAACAAAA TATTAACGTT 2250

TACAATTTTA TGGTGCACTC TCAGTACAAT CTGCTCTGAT GCCGCATAGT 2300

TAAGCCAACT CCGCTATCGC TACGTGACTG GGTCATGGCT GCGCCCCGAC 2350

30 ACCCGCCAAC ACCCGCTGAC GCGCCCTGAC GGGCTTGTCT GCTCCCGGCA 2400

TCCGCTTACA GACAAGCTGT GACCGTCTCC GGGAGCTGCA TGTGTCAGAG 2450

35 GTTTTCACCG TCATCACCGA AACGCGCGAG GCAGTATTCT TGAAGACGAA 2500

40 AGGGCCTCGT GATACGCCTA TTTTATAGG TTAATGTCAT GATAATAATG 2550

GTTTCTTAGA CGTCAGGTGG CACTTTTCGG GGAAATGTGC GCGGAACCCC 2600

45 TATTTGTTTA TTTTCTAAA TACATTCAA TATGTATCCG CTCATGAGAC 2650

AATAACCCTG ATAAATGCTT CAATAATATT GAAAAAGGAA GAGTATGAGT 2700

50 ATTCAACATT TCCGTGTCGC CCTTATTCCC TTTTTCGCGG CATTTTGCCT 2750

55 TCCTGTTTTT GCTCACCCAG AAACGCTGGT GAAAGTAAAA GATGCTGAAG 2800

ATCAGTTGGG TGCACGAGTG GGTACATCG AACTGGATCT CAACAGCGGT 2850

5 AAGATCCTTG AGAGTTTTTCG CCCCGAAGAA CGTTTTCCAA TGATGAGCAC 2900

TTTTAAAGTT CTGCTATGTG GCGCGGTATT ATCCCGTGAT GACGCCGGGC 2950

10 AAGAGCAACT CGGTCGCCGC ATACACTATT CTCAGAATGA CTTGGTTGAG 3000

TACTCACCAG TCACAGAAAA GCATCTTACG GATGGCATGA CAGTAAGAGA 3050

15 ATTATGCAGT GCTGCCATAA CCATGAGTGA TAACACTGCG GCCAACTTAC 3100

TTCTGACAAC GATCGGAGGA CCGAAGGAGC TAACCGCTTT TTTGCACAAC 3150

20 ATGGGGGATC ATGTAACTCG CCTTGATCGT TGGGAACCGG AGCTGAATGA 3200

AGCCATACCA AACGACGAGC GTGACACCAC GATGCCAGCA GCAATGGCAA 3250

CAACGTTGCG CAACTATTA ACTGGCGAAC TACTTACTCT AGCTTCCCGG 3300

30 CAACAATTAA TAGACTGGAT GGAGGCGGAT AAAGTTGCAG GACCACTTCT 3350

GCGCTCGGCC CTTCCGGCTG GCTGGTTTAT TGCTGATAAA TCTGGAGCCG 3400

35 GTGAGCGTGG GTCTCGCGGT ATCATTGCAG CACTGGGGCC AGATGGTAAG 3450

CCCTCCCGTA TCGTAGTTAT CTACACGACG GGGAGTCAGG CAACTATGGA 3500

TGAACGAAAT AGACAGATCG CTGAGATAGG TGCCTCACTG ATTAAGCATT 3550

45 GGTAAGTGTG AGACCAAGTT TACTCATATA TACTTTAGAT TGATTTAAAA 3600

CTTCATTTTT AATTTAAAAG GATCTAGGTG AAGATCCTTT TTGATAATCT 3650

50 CATGACCAAA ATCCCTTAAC GTGAGTTTTT GTTCCACTGA GCGTCAGACC 3700

55 CCGTAGAAAA GATCAAAGGA TCTTCTTGAG ATCCTTTTTT TCTGCGCGTA 3750

ATCTGCTGCT TGCAAACAAA AAAACCACCG CTACCAGCGG TGGTTTGT TT 3800

5 GCCGGATCAA GAGCTACCAA CTCCTTTTCC GAAGGTA ACT GGCTTCAGCA 3850

GAGCGCAGAT ACCAAATACT GTCCTTCTAG TGTAGCCGTA GTTAGGCCAC 3900

10 CACTTCAAGA ACTCTGTAGC ACCGCCTACA TACCTCGCTC TGCTAATCCT 3950

GTTACCAGTG GCTGCTGCCA GTGGCGATAA GTCGTGTCTT ACCGGGTTGG 4000

15 ACTCAAGACG ATAGTTACCG GATAAGGCGC AGCGGTCGGG CTGAACGGGG 4050

GGTTCGTGCA CACAGCCCAG CTTGGAGCGA ACGACCTACA CCGAACTGAG 4100

20 ATACCTACAG CGTGAGCATT GAGAAAGCGC CACGCTTCCC GAAGGGAGAA 4150

AGGCGGACAG GTATCCGGTA AGCGGCAGGG TCGGAACAGG AGAGCGCACG 4200

AGGGAGCTTC CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTCGGGTT 4250

30 TCGCCACCTC TGACTTGAGC GTCGATTTT GTGATGCTCG TCAGGGGGGC 4300

GGAGCCTATG GAAAAACGCC AGCAACGCGG CCTTTTACG GTTCCTGGCC 4350

35 TTTTGCTGGC CTTTGCTCA CATGTTCTT CCTGCGTTAT CCCCTGATTC 4400

40 TGTGGATAAC CGTATTACCG CCTTTGAGTG AGCTGATACC GCTCGCCGCA 4450

GCCGAACGAC CGAGCGCAGC GAGTCAGTGA GCGAGGAAGC GGAAGAGCGC 4500

45 CCAATACGCA AACCGCCTCT CCCCGCGCGT TGGCCGATTC ATTAATCCAG 4550

CTGGCACGAC AGGTTTCCCG ACTGGAAAGC GGGCAGTGAG CGCAACGCAA 4600

50 TTAATGTGAG TTACCTCACT CATTAGGCAC CCCAGGCTTT AACTTTATG 4650

55 CTTCCGGCTC GTATGTTGTG TGAATTGTG AGCGGATAAC AATTCACAC 4700

AGGAAACAGC TATGACCATG ATTACGAATT AA 4732

5 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 47 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

15

TTGGAATCCC ATTTACAACC TCGAGTTGTT TCGTTTTGGC ACAAGAT 47

20 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

30

GAATCCCAT TACGACGTCC AATTGTTTCG 30

35 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 26 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

45

CCCATTTACA ACTGCCAATT GTTTTCG 26

50 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 55 (A) LENGTH: 22 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

5 AGAAGGGAAA CAGTGTCGTG CA 22

(2) INFORMATION FOR SEQ ID NO:6:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

20 AGTGGGCCAC CAGAATCCCC CT 22

(2) INFORMATION FOR SEQ ID NO:7:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

35 TCCACGACCA GGAGAAATGA CAC 23

(2) INFORMATION FOR SEQ ID NO:8:

40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
45 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

50 GCATTCAACT TCTGAGTTTC TAATGTAGTC 30

(2) INFORMATION FOR SEQ ID NO:9:

55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CATAGTATTG TCAGCTTCAA CTTCTGAACA 30

10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 30 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCCATGTGAC ATATCTTCAG TTGTTTCCAA 30

25

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 30 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGTGGTATCA CCTTCATCTT GTCCATGTGA 30

40

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 24 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACCTTGGATG CATTAGTTG TTTC 24

55

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 23 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

10

TTGTCCATGT GATTAATCAC AGT 23

15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 35 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

25

GTTCGTGTTG GGATCCCATTT TACCTATCGC AATTG 35

30

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 10596 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

40

TTCGAGCTCG CCCGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT 50

45

TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC GTTACATAAC 100

TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTTG 150

50

ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA 200

55

TTGACGTCAA TGGGTGGAGT ATTTACGGTA AACTGCCCAC TTGGCAGTAC 250

ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT CAATGACGGT 300

5 AAATGGCCCG CCTGGCATTG TGCCCAGTAC ATGACCTTAT GGGACTTTCC 350

TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC 400

10 GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA 450

TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA 500

15 AAATCAACGG GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC 550

AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG CAGAGCTCGT 600

20 TTAGTGAACC GTCAGATCGC CTGGAGACGC CATCCACGCT GTTTTGACCT 650

CCATAGAAGA CACCGGGACC GATCCAGCCT CCGCGGCCGG GAACGGTGCA 700

25 TTGGAACGCG GATTCCCCGT GCCAAGAGTG ACGTAAGTAC CGCCTATAGA 750

30 GTCTATAGGC CCACCCCTT GGCTTCGTTA GAACGCGGCT ACAATTAATA 800

CATAACCTTA TGTATCATAC ACATACGATT TAGGTGACAC TATAGAATAA 850

35 CATCCAETTT GCCTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACCTGC 900

ACCTCGGTTT TATCGATTCT CGAGAATTAA TTCAAGCTTG CGGCCGCAGC 950

40 TTGGCCGCCA TGGCCCAACT TGTTTATTGC AGCTTATAAT GGTACAAAT 1000

45 AAAGCAATAG CATCACAAAT TTCACAAATA AAGCATTTTT TTCACTGCAT 1050

TCTAGTTGTG GTTTGTCCAA ACTCATCAAT GTATCTTATC ATGTCTGGAT 1100

50 CGATCGGGAA TTAATTCGGC GCAGCACCAT GGCCTGAAAT AACCTCTGAA 1150

55 AGAGGAACCTT GGTTAGGTAC CTTCTGAGGC GGAAAGAACC AGCTGTGGAA 1200

TGTGTGTCAG TTAGGGTGTG GAAAGTCCCC AGGCTCCCCA GCAGGCAGAA 1250

5 GTATGCAAAG CATGCATCTC AATTAGTCAG CAACCAGGTG TGGAAAGTCC 1300

CCAGGCTCCC CAGCAGGCAG AAGTATGCAA AGCATGCATC TCAATTAGTC 1350

10 AGCAACCATA GTCCCGCCCC TAACTCCGCC CATCCCGCCC CTAACTCCGC 1400

CCAGTTCCGC CCATTCTCCG CCCCATGGCT GACTAATTTT TTTTATTTAT 1450

15 GCAGAGGCCG AGGCCGCCTC GGCCTCTGAG CTATTCCAGA AGTAGTGAGG 1500

AGGCTTTTTT GGAGGCCTAG GCTTTTGCAA AAAGCTGTTC ACGTGATGAA 1550

20 TTCTCATGTT TGACAGCTTA TCATCGATAG ATCCTCACAG GCCGCACCCA 1600

GCTTTTCTTC CGTTGCCCA GTAGCATCTC TGTCTGGTGA CCTTGAAGAG 1650

25 GAAGAGGAGG GGTCCCGAGA ATCCCATCC CTACCGTCCA GCAAAAAGGG 1700

30 GGACGAGGAA TTGAGGCCT GGCTTGAGGC TCAGGACGCA AATCTTGAGG 1750

ATGTTGAGCG GGAGTTTTCC GGGCTGCGAG TAATTGGTGA TGAGGACGAG 1800

35 GATGGTTCGG AGGATGGGGA ATTTTCAGAC CTGGATCTGT CTGACAGCGA 1850

CCATGAAGGG GATGAGGGTG GGGGGGCTGT TGGAGGGGGC AGGAGTCTGC 1900

ACTCCCTGTA TTCACTGAGC GTCGTCTAAT AAAGATGTCT ATTGATCTCT 1950

45 TTTAGTGTGA ATCATGTCTG ACGAGGGGCC AGGTACAGGA CCTGGAAATG 2000

GCCTAGGAGA GAAGGGAGAC ACATCTGGAC CAGAAGGCTC CGGCGGCAGT 2050

50 GGACCTCAA GAAGAGGGGG TGATAACCAT GGACGAGGAC GGGGAAGAGG 2100

55 ACGAGGACGA GGAGGCGGAA GACCAGGAGC CCCGGGCGGC TCAGGATCAG 2150

GGCCAAGACA TAGAGATGGT GTCCGGAGAC CCCAAAACG TCCAAGTTGC 2200

5 ATTGGCTGCA AAGGGACCCA CGGTGGAACA GGAGCAGGAG CAGGAGCGGG 2250

AGGGGCAGGA GCAGGAGGGG CAGGAGCAGG AGGAGGGGCA GGAGCAGGAG 2300

10 GAGGGGCAGG AGGGGCAGGA GGGGCAGGAG GGGCAGGAGC AGGAGGAGGG 2350

GCAGGAGCAG GAGGAGGGGC AGGAGGGGCA GGAGGGGCAG GAGCAGGAGG 2400

15 AGGGGCAGGA GCAGGAGGAG GGGCAGGAGG GGCAGGAGCA GGAGGAGGGG 2450

CAGGAGGGGC AGGAGGGGCA GGAGCAGGAG GAGGGGCAGG AGCAGGAGGA 2500

20 GGGGCAGGAG GGGCAGGAGC AGGAGGAGGG GCAGGAGGGG CAGGAGGGGC 2550

25 AGGAGCAGGA GGAGGGGCAG GAGCAGGAGG GGCAGGAGGG GCAGGAGGGG 2600

CAGGAGCAGG AGGGGCAGGA GCAGGAGGAG GGGCAGGAGG GGCAGGAGGG 2650

30 GCAGGAGCAG GAGGGGCAGG AGCAGGAGGG GCAGGAGCAG GAGGGGCAGG 2700

AGCAGGAGGG GCAGGAGGGG CAGGAGCAGG AGGGGCAGGA GGGGCAGGAG 2750

35 CAGGAGGGGC AGGAGGGGCA GGAGCAGGAG GAGGGGCAGG AGGGGCAGGA 2800

40 GCAGGAGGAG GGGCAGGAGG GGCAGGAGCA GGAGGGGCAG GAGGGGCAGG 2850

AGCAGGAGGG GCAGGAGGGG CAGGAGCAGG AGGGGCAGGA GGGGCAGGAG 2900

45 CAGGAGGAGG GGCAGGAGCA GGAGGGGCAG GAGCAGGAGG TGGAGGCCGG 2950

GGTCGAGGAG GCAGTGGAGG CCGGGGTCGA GGAGGTAGTG GAGGCCGGGG 3000

50 TCGAGGAGGT AGTGGAGGCC GCCGGGGTAG AGGACGTGAA AGAGCCAGGG 3050

55 GGGGAAGTCG TGAAAGAGCC AGGGGGAGAG GTCGTGGACG TGGAGAAAAG 3100

AGGCCCAGGA GTCCCAGTAG TCAGTCATCA TCATCCGGGT CTCCACCGCG 3150

5 CAGGCCCCCT CCAGGTAGAA GGCCATTTT CCACCCTGTA GGGGAAGCCG 3200

ATTATTTTGA ATACCACCAA GAAGGTGGCC CAGATGGTGA GCCTGACGTG 3250

10 CCCCCGGGAG CGATAGAGCA GGGCCCCGCA GATGACCCAG GAGAAGGCCC 3300

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15 GGTGGTTTGG AAAGCATCGT GGTCAAGGAG GTTCCAACCC GAAATTTGAG 3400

AACATTGCAG AAGGTTTAAG AGCTCTCCTG GCTAGGAGTC ACGTAGAAAG 3450

20 GACTACCGAC GAAGGAACTT GGGTCGCCGG TGTGTTTCGTA TATGGAGGTA 3500

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CAATGTCGTC TTACACCATT GAGTCGTCTC CCCTTTGGAA TGGCCCCTGG 3600

30 ACCCGGCCCA CAACCTGGCC CGCTAAGGGA GTCCATTGTC TGTATTTCAT 3650

TGGTCTTTTT ACAAACTCAT ATATTTGCTG AGGTTTGGAA GGATGCGATT 3700

35 AAGGACCTTG TTATGACAAA GCCCCTCCT ACCTGCAATA TCAGGGTGAC 3750

TGTGTGCAGC TTTGACGATG GAGTAGATTT GCCTCCCTGG TTTCCACCTA 3800

TGGTGGAAGG GGCTGCCGCG GAGGGTGATG ACGGAGATGA CGGAGATGAA 3850

45 GGAGGTGATG GAGATGAGGG TGAGGAAGGG CAGGAGTGAT GTAACCTGTT 3900

AGGAGACGCC CTCAATCGTA TTAAAAGCCG TGTATTCCCC CGCACTAAAG 3950

50 AATAAATCCC CAGTAGACAT CATGCGTGCT GTTGGTGTAT TTCTGGCCAT 4000

55 CTGTCTTGTC ACCATTTTCG TCCTCCCAAC ATGGGGCAAT TGGGCATACC 4050

CATGTTGTCA CGTCACTCAG CTCCGCGCTC AACACCTTCT CGCGTTGGAA 4100

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10 AAGGACAAGC AGCGAAAATT CACGCCCCCT TGGGAGGTGG CGGCATATGC 4250

AAAGGATAGC ACTCCCACTC TACTACTGGG TATCATATGC TGA CTGTATA 4300

15 TGCATGAGGA TAGCATATGC TACCCGGATA CAGATTAGGA TAGCATATAC 4350

TACCCAGATA TAGATTAGGA TAGCATATGC TACCCAGATA TAGATTAGGA 4400

20 TAGCCTATGC TACCCAGATA TAAATTAGGA TAGCATATAC TACCCAGATA 4450

TAGATTAGGA TAGCATATGC TACCCAGATA TAGATTAGGA TAGCCTATGC 4500

TACCCAGATA TAGATTAGGA TAGCATATGC TACCCAGATA TAGATTAGGA 4550

30 TAGCATATGC TATCCAGATA TTTGGGTAGT ATATGCTACC CAGATATAAA 4600

TTAGGATAGC ATATACTACC CTAATCTCTA TTAGGATAGC ATATGCTACC 4650

35 CGGATACAGA TTAGGATAGC ATATACTACC CAGATATAGA TTAGGATAGC 4700

40 ATATGCTACC CAGATATAGA TTAGGATAGC CTATGCTACC CAGATATAAA 4750

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55 CTTGGCGCTC AGGCGCAAGT GTGTGTAATT TGTCCTCCAG ATCGCAGCAA 5000

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15 GTTTAATTTT CGGGGGTGTT AGAGACAACC AGTGGAGTCC GCTGCTGTCTG 5300

GCGTCCACTC TCTTFCCTT TGTTACAAAT AGAGTGTAAC AACATGGTTC 5350

20 ACCTGTCTTG GTCCCTGCCT GGGACACATC TTAATAACCC CAGTATCATA 5400

25 TTGCACTAGG ATTATGTGTT GCCCATAGCC ATAAATTCGT GTGAGATGGA 5450

CATCCAGTCT TTACGGCTTG TCCCCACCCC ATGGATTTCT ATTGTTAAAG 5500

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35 CCAAATTTTA TTCTGGGGGC GTCACCTGAA ACCTTGTTTT CGAGCACCTC 5650

40 ACATACACCT TACTGTTTAC AACTCAGCAG TTATTCTATT AGCTAAACGA 5700

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20 AGCTCTTCAG CAATATCAGC GGTCAGCCAA GCTATGTCCT GATAGCGGTC 7300

25 CGCCACACCC AGCCGGCCAC AGTCGATGAA TCCAGAAAAG CGGCCATTTT 7350

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35 CCATCCGAGT ACGTGCTCGC TCGATGCGAT GTTTCGCTTG GTGGTCGAAT 7550

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15 GTGTTTGAAT TCATCAAAGC AACCATAGTA CGCGCCCTGT AGCGGCGCAT 8150

TAAGCGCGGC GGGTGTGGTG GTTACGCGCA GCGTGACCGC TACACTTGCC 8200

20 AGCGCCCTAG CGCCCGCTCC TTTCGCTTTC TTCCCTTCCT TTCTCGCCAC 8250

25 GTTCGCCGGC TTCCCCGTC AAGCTCTAAA TCGGGGGCTC CCTTTAGGGT 8300

TCCGATTTAG TGCTTTACGG CACCTCGACC CCAAAAACT TGATTTGGGT 8350

30 GATGGTTCAC GTAGTGGGCC ATCGCCCTGA TAGACGGTTT TTCGCCCTTT 8400

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35 CAACACTCAA CCCTATCTCG GGCTATTCTT TTGATTTATA AGGGATTTTG 8500

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30 CAAACGACGA GCGTGACACC ACGATGCCAG CAGCAATGGC AACAACGTTG 9350

CGCAAATAT TAACTGGCGA ACTACTTACT CTAGCTTCCC GGCAACAATT 9400

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40 CCCTTCCGGC TGGCTGGTTT ATTGCTGATA AATCTGGAGC CGGTGAGCGT 9500

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45 TATCGTAGTT ATCTACACGA CGGGGAGTCA GGCAACTATG GATGAACGAA 9600

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50 TCAGACCAAG TTTACTCATA TATACTTTAG ATTGATTAA AACTTCATTT 9700

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10 AAGAGCTACC AACTCTTTTT CCGAAGGTAA CTGGCTTCAG CAGAGCGCAG 9950
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15 GAACTCTGTA GCACCGCCTA CATACTCGC TCTGCTAATC CTGTTACCAG 10050
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30 AGGTATCCGG TAAGCGGCAG GGTCGGAACA GGAGAGCGCA CGAGGGAGCT 10300
TCCAGGGGGA AACGCCTGGT ATCTTTATAG TCCTGTCGGG TTTCGCCACC 10350
35 TCTGACTTGA GCGTCGATTT TTGTGATGCT CGTCAGGGGG GCGGAGCCTA 10400
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40 TGAGCGCAAC GCAATTAATG TGAGTTACCT CACTCATTAG GCACCCAGG 10500
45 CTTTACACTT TATGCTTCCG GCTCGTATGT TGTGTGGAAT TGTGAGCGGA 10550
TAACAATTTT ACACAGGAAA CAGCTATGAC CATGATTACG AATTAA 10596
50

(2) INFORMATION FOR SEQ ID NO:16:

55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 bases

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATGAAGGCCC CCGCTGTGCT TGCACCTGGC ATCCTCGTGC TCCTGTTTAC 50

10

C 51

15 (2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 56 bases

(B) TYPE: nucleic acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

25

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CATGAA 56

30

(2) INFORMATION FOR SEQ ID NO:18:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TAGTACTAGC ACTATGATGT CT 22

45

(2) INFORMATION FOR SEQ ID NO:19:

50 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

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5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 25 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

15

CAGGGGGAGT TGCAGATTCA GCTGT 25

20

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 45 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

30

AGTTTTGTCG GTGACCTGAT CATTCTGATC TGGTTGAACT ATTAC 45

35

Claims:

1. A hepatocyte growth factor (HGF) variant resistant to proteolytic cleavage by enzymes that are capable of in vivo conversion of HGF into its two-chain form.
- 5 2. The variant of claim 1 which is a variant of human HGF (huHGF):
3. A hepatocyte growth factor (HGF) variant stabilized in single-chain form by site directed mutagenesis within a region recognized by an enzyme capable of converting HGF into its two-chain
10 form.
4. The variant of claim 3 which is capable of binding an HGF receptor.
5. The variant of claim 4 which is a variant of human HGF (huHGF).
- 15 6. The variant of claim 5 having an amino acid alteration at or adjacent to amino acid positions 493, 494, 495 or 496 of the wild-type huHGF amino acid sequence.
7. The variant of claim 6 wherein said alteration is substitution.
- 20 8. The variant of claim 6 wherein said alteration is insertion or deletion.
9. The variant of claim 6 in which amino acid position 494 is occupied by an amino acid other than arginine.
10. The variant of claim 9 wherein said amino acid is selected
25 from the group consisting of glutamic acid, aspartic acid and alanine.
11. The variant of claim 6 wherein said alteration is the substitution of at least one amino acid at amino acid positions 493-496 of the wild-type hHGF amino acid sequence.
12. The variant of claim 11 having another amino acid
30 substituted for arginine at amino acid position 494 of wild-type huHGF.
13. The variant of claim 11 having another amino acid substituted for valine at amino acid position 495 of wild-type huHGF.
14. The variant of claim 11 having another amino acid
35 substituted for valine at amino acid position 496 of wild-type huHGF.
15. The variant of claim 6 retaining substantially full receptor binding affinity of wild-type huHGF.

16. A hepatocyte growth factor (HGF) variant having an amino acid alteration at a site within the protease domain of HGF and retaining substantially full receptor binding affinity of the corresponding wild-type HGF.
- 5 17. The variant of claim 16 comprising an alteration in a region corresponding to the catalytic site of serine proteases.
18. The variant of claim 16 comprising an alteration at or adjacent to any of positions 534, 673 and 692 of the wild-type human HGF (huHGF) amino acid sequence.
- 10 19. The variant of claim 18 wherein said alteration is substitution.
20. The variant of claim 19 having another amino acid substituted for glutamine at position 534 of the wild-type huHGF amino acid sequence.
- 15 21. The variant of claim 20 wherein said amino acid is histidine.
22. The variant of claim 19 having another amino acid substituted for tyrosine at position 673 of the wild-type huHGF amino acid sequence.
- 20 23. The variant of claim 22 wherein said amino acid is devoid of aromatic and heterocyclic moieties.
24. The variant of claim 23 wherein said amino acid is selected from serine, threonine, asparagine, cysteine, glycine, alanine, and valine.
- 25 25. The variant of claim 24 wherein said amino acid is serine.
26. The variant of claim 19 having another amino acid substituted for valine at position 692 of the wild-type huHGF amino acid sequence.
- 30 27. The variant of claim 26 wherein said amino acid is polar.
28. The variant of claim 27 wherein said amino acid is selected from serine, threonine, asparagine and glutamine.
29. The variant of claim 28 wherein said amino acid is serine.
30. The variant of claim 24 further comprising the substitution
- 35 of glutamine at position 534 or valine at position 692 of the wild-type huHGF amino acid sequence.

31. The variant of claim 30 having serine substituted for tyrosine at position 673 of the wild-type huHGF amino acid sequence.
32. The variant of claim 31 having histidine substituted for glutamine at position 534 of the wild-type huHGF amino acid sequence.
- 5 33. The variant of claim 31 having serine substituted for valine at position 692 of the wild-type huHGF amino acid sequence.
34. The variant of claim 33 additionally having histidine substituted for glutamine at position 534 of the wild-type huHGF amino acid sequence.
- 10 35. The variant of any of claims 1-15 having an amino acid alteration at a site within the protease domain of HGF and retaining substantially full receptor binding affinity of the corresponding wild-type HGF.
36. The variant of any of claims 16-34 that is resistant to
- 15 proteolytic cleavage.
37. The variant of any of claims 1-36 that is substantially incapable of HGF receptor activation.
38. The variant of any of claims 1-36 that is substantially devoid of HGF hepatocyte growth stimulating activity.
- 20 39. The variant of any of claims 1-36 having increased receptor binding affinity as compared to wild-type huHGF.
40. The variant of claim 39 wherein the increase in receptor binding affinity is accomplished by an alteration in a receptor-binding domain of the huHGF amino acid sequence.
- 25 41. The variant of claim 40 wherein said alteration is in the huHGF α -chain.
42. The variant of claim 41 wherein said alteration is within the Kringle 1 domain.
43. The variant of claim 42 wherein said alteration is within
- 30 the patch defined by amino acid positions 159, 161, 195 and 197 of the wild-type huHGF amino acid sequence.
44. The variant of claim 42 wherein said alteration is at amino acid position 173 of wild-type huHGF.
45. The variant of claim 41 wherein said alteration is within
- 35 the hairpin domain, N-terminal of the hairpin domain, or between the hairpin and the Kringle 1 domains of wild-type huHGF.

46. The variant of any of claims 1-45 devoid of functional Kringle 2 domain.

47. The variant of any of claims 1-45 devoid of functional Kringle 3 domain.

5 48. The variant of any of claims 1-45 devoid of functional Kringle 4 domain.

49. A nucleotide sequence encoding the variant of any of claims 1-48.

10 50. A replicable expression vector containing and capable of expressing in a suitable host cell the nucleotide sequence of claim 49.

51. A host cell transformed with the vector of claim 50.

52. A process comprising culturing the host cells of claim 51 so as to express the nucleic acid encoding the HGF variant.

15 53. The process of claim 52 further comprising recovering the variant from the host cell culture.

54. A pharmaceutical composition comprising a variant of any of claims 1-48 in an amount capable of competitive inhibition of the binding of wild-type huHGF to its receptor, in admixture with a pharmaceutically acceptable carrier.

20 55. A method of treating a pathological condition associated with the activation of a huHGF receptor comprising administering to a patient in need the composition of claim 54.

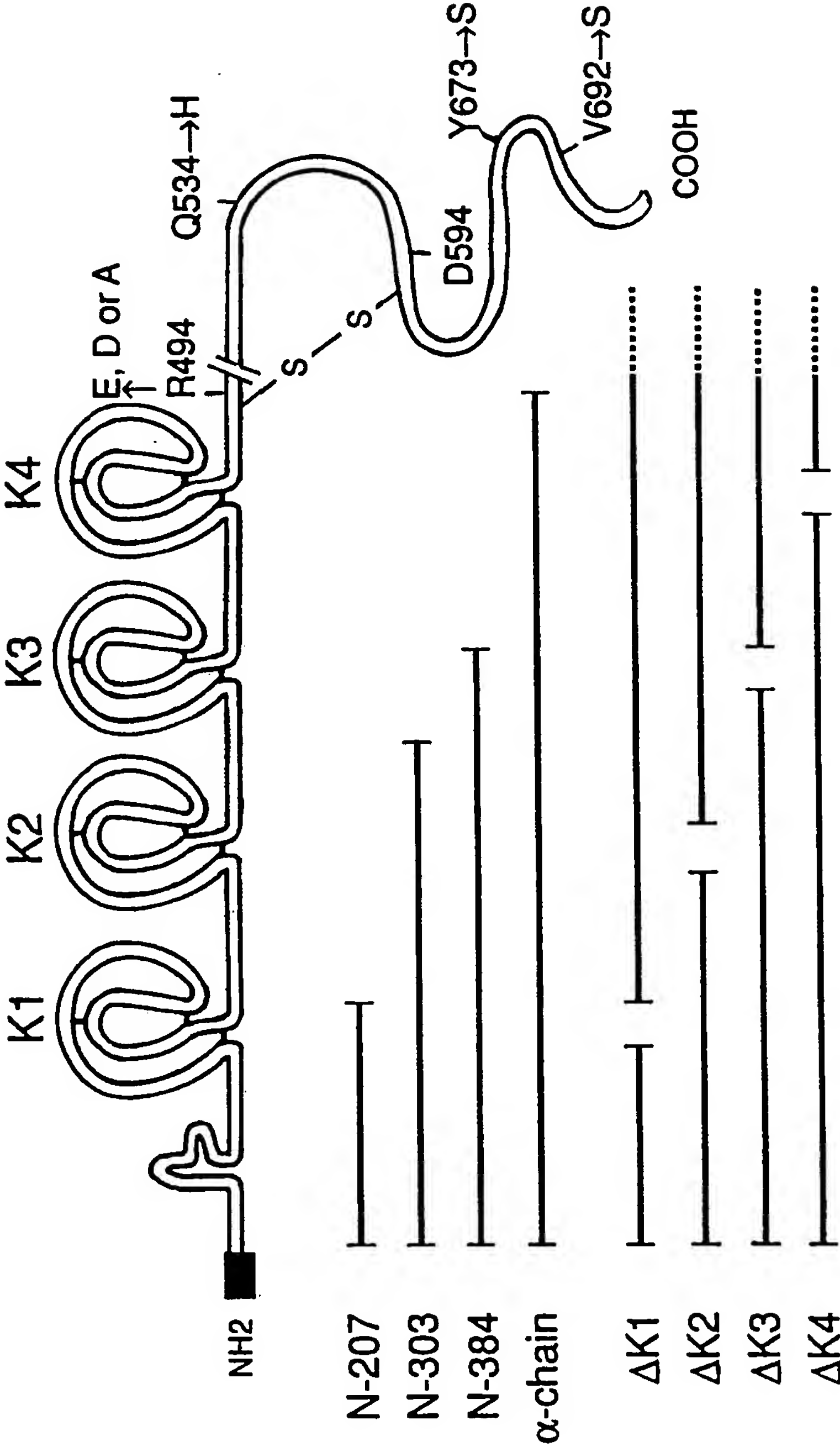


FIG. 1

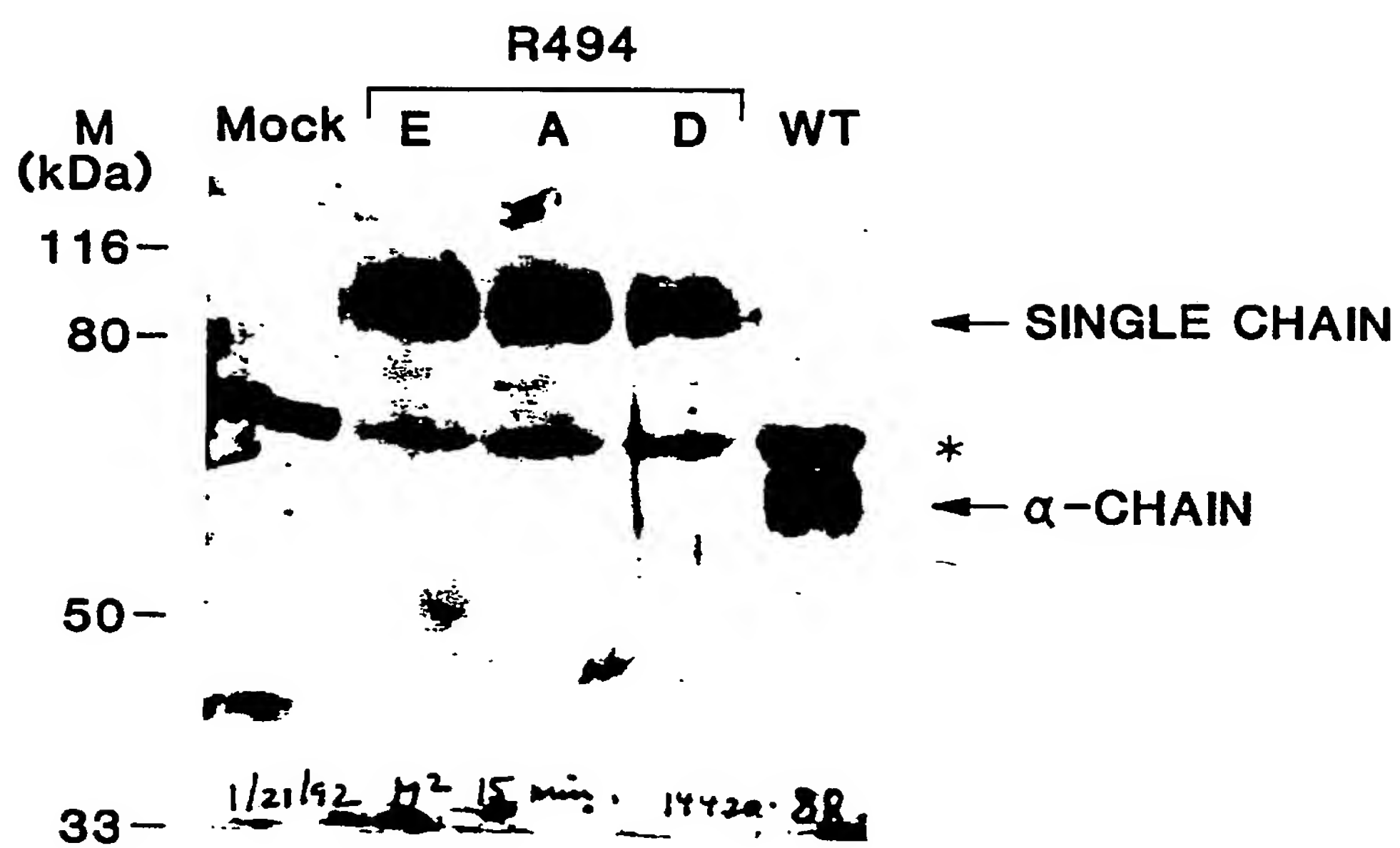


FIG. 2

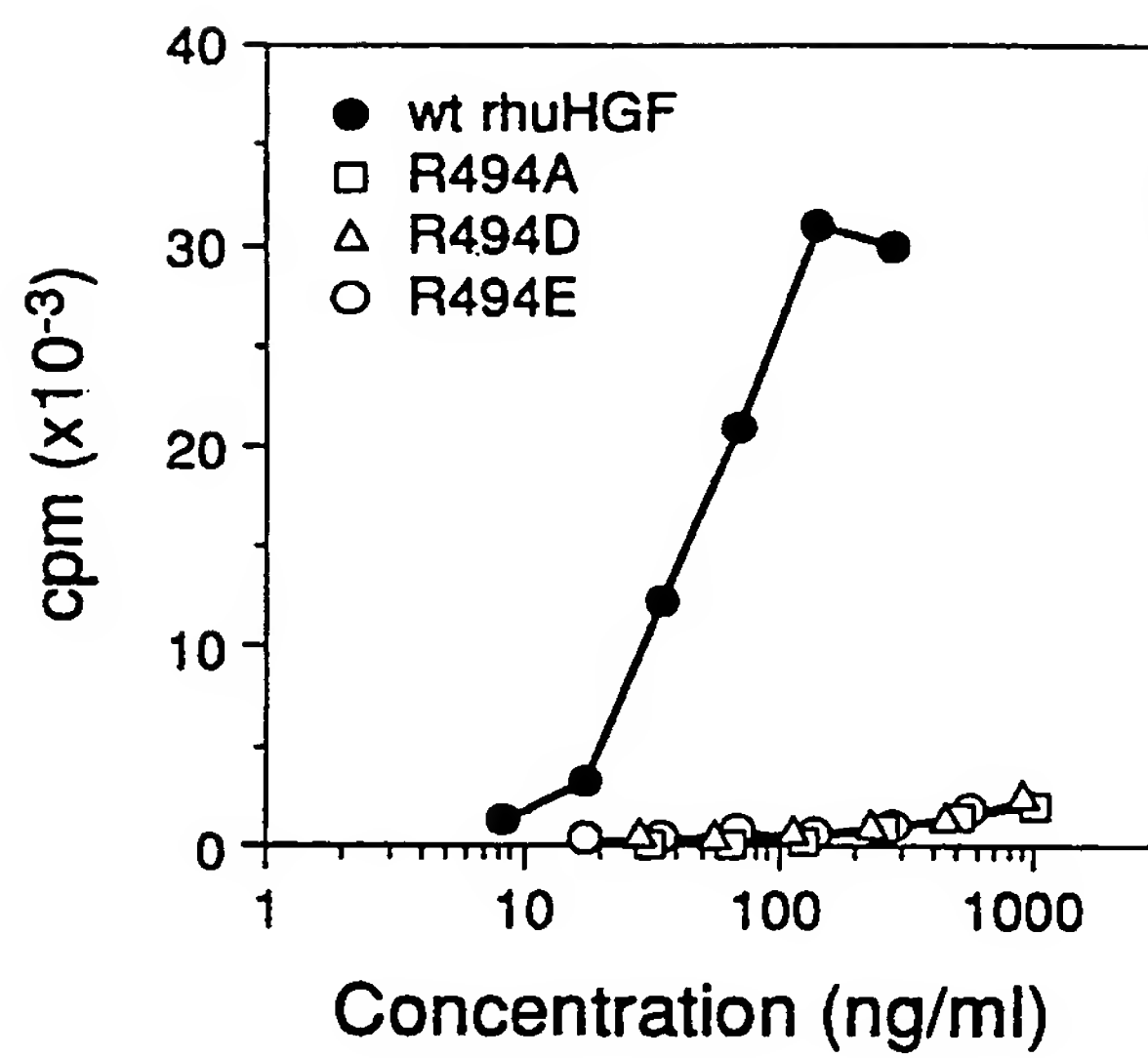


FIG. 3A

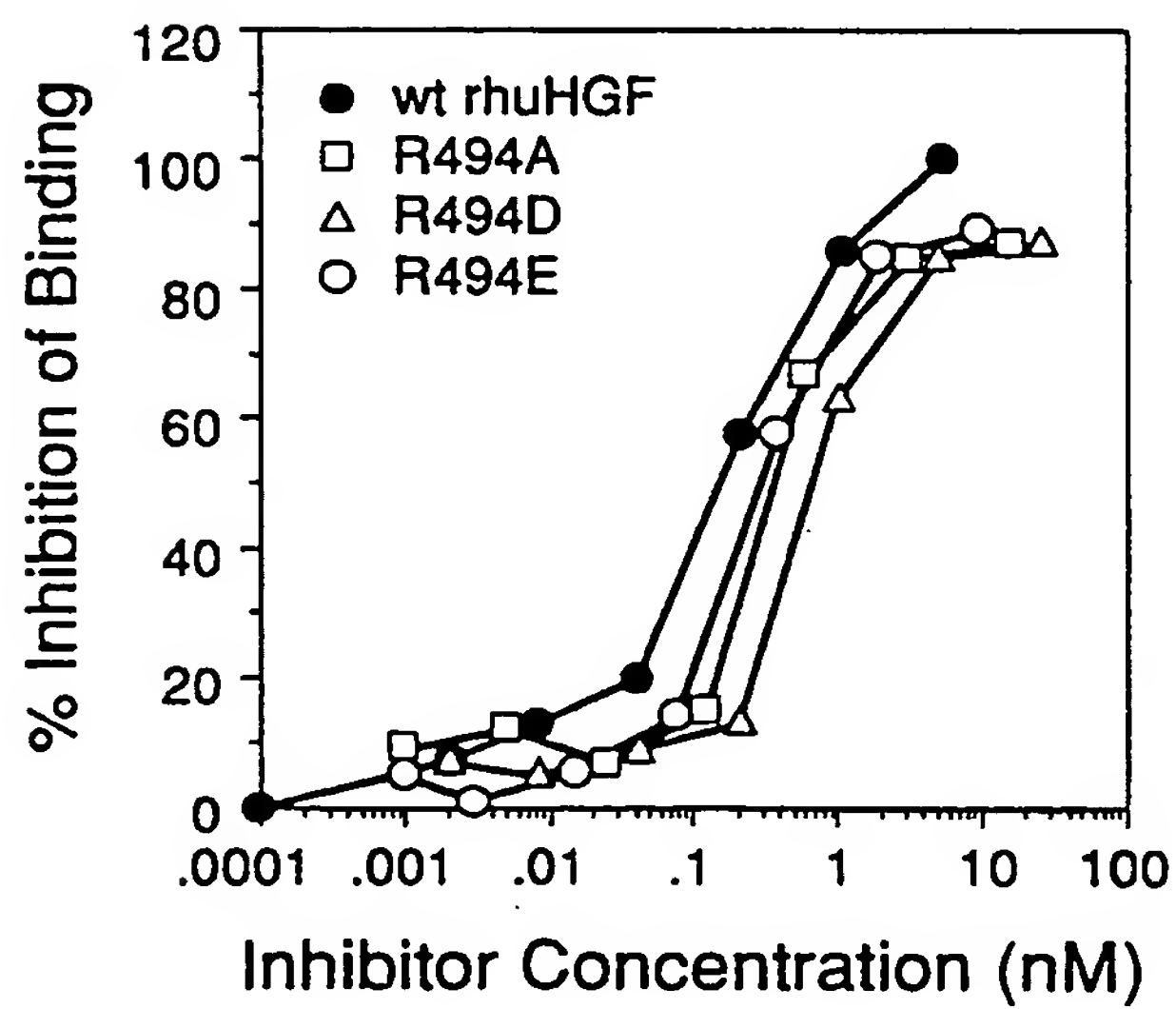


FIG. 3B

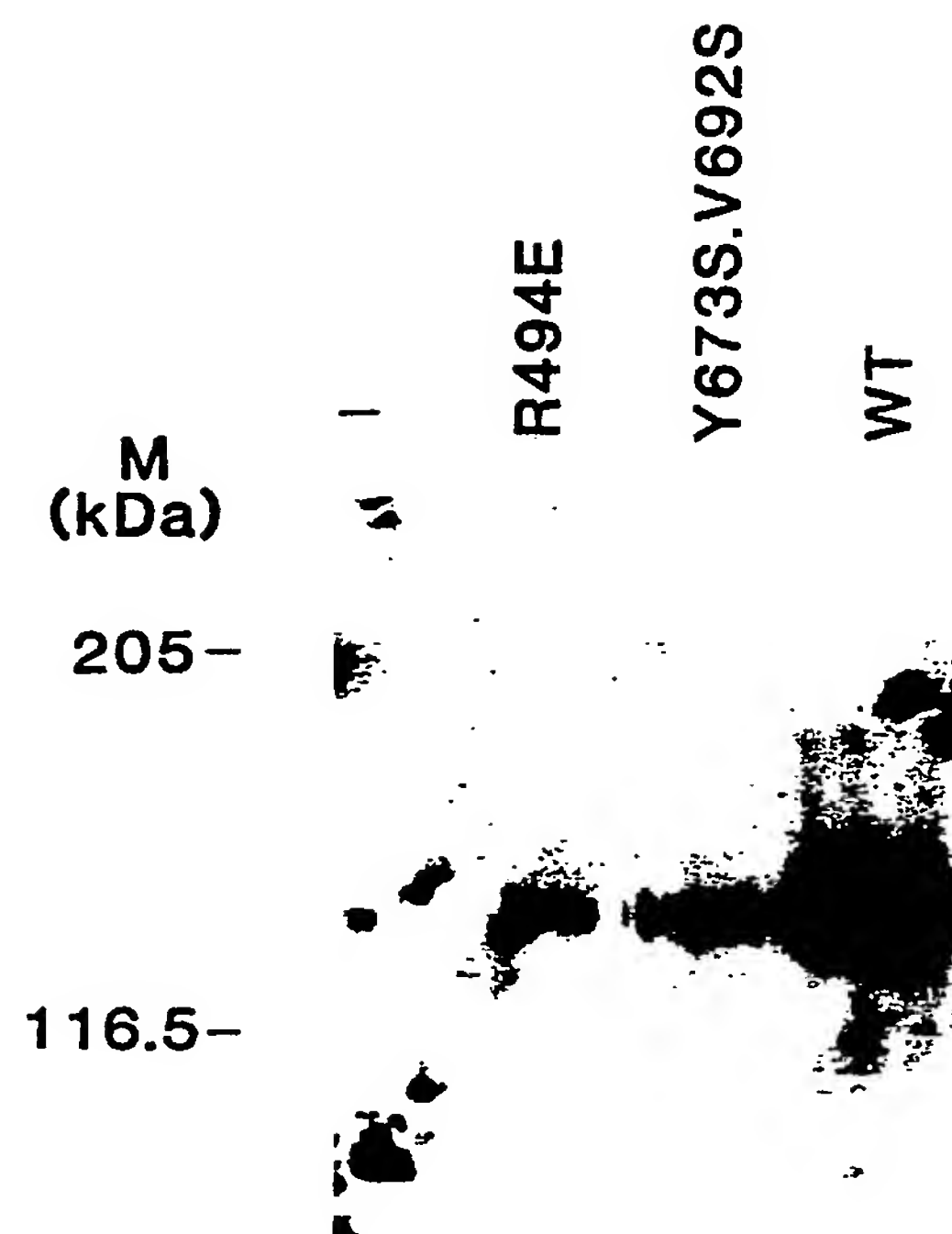


FIG. 4


```

><Modified RK5 derivative, insert Xho I into Hind III site; PJG
2/89>
<from Cori 86.3.18 fix DHFR acc I
<sequence of CMV enhancer/promoter, from Cell 41, 1985>
T><from pPMLCMV beginning to HindIII, enhancers and promoter>
TCGAGCTCGCCCGACATTGATTA
TTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCC
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```

FIG. 5A

GCGAAGAGGCCCCGCACCGATCGCCCTTCCCAACAGTTGCGTAGCCTGAATGGCGAATGGC
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 G CCTGATGCGG
 TATTTTCTCC TTACGCATCT GTGCGGTATT TCACACCGCA TA

 CGTCAAAGCAACCATAGTACGCGCCCTGTAGCGGCGCATTAAAGCGCGGGCGGGTG
 TGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTTCG
 CTTTCTTCCCTTCCTTTCTCGCCACGTTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGG
 GGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATT
 TGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGT
 TGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTA
 TCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTTCGGCCTATTGGTTAAAAA
 ATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTT

 TATGGTGCAC TCTCAGTACA
 ATCTGCTCTG ATGCCGCATA GTTAAGCCA ACTCC GCTATCGCTA
 CGTGACTGGG
 TCATGGCTGC GCCCCGACAC CCGCCAACAC CCGCTGACGC GCCCTGACGG
 GCTTGTCTGC
 TCCCGGCATC CGCTTACAGA CAAGCTGTGA CCGTCTCCGG GAGCTGCATG
 TGTCAGAGGT
 TTTCACCGTC ATCACCGAAA CGCGCGAGGC AG
 TATTC
 < Hinc II (2271) to GTCATC>
 < Pst I (1973) to CTGCTG>
 < Acc I (183) delete 6 bp>
 <Arbitrarily change EcoRI (1) to GAATAC>
 <pUCx 83.11.25 sequence not fully known>
 TTGAAGACGA AAGGGCCTCG TGATACGCCT ATTTTATAG
 GTTAATGTCA TGATAATAAT GGTTCCTTAG ACGTCAGGTG GCACTTTTTCG
 GGGAAATGTG
 CGCGGAACCC CTATTTGTTT ATTTTCTAA ATACATTCAA ATATGTATCC
 GCTCATGAGA
 CAATAACCCT GATAAATGCT TCAATAATAT TGAAAAAGGA AGAGTATGAG
 TATTCAACAT
 TTCCGTGTCTG CCCTTATTCC CTTTTTTGCG GCATTTTGCC TTCCTGTTTT
 TGCTCACCCA
 GAAACGCTGG TGAAAGTAAA AGATGCTGAA GATCAGTTGG GTGCACGAGT
 GGGTTACATC
 GAACTGGATC TCAACAGCGG TAAGATCCTT GAGAGTTTTC GCCCCGAAGA
 ACGTTTTTCCA
 ATGATGAGCA CTTTTAAAGT TCTGCTATGT GGCGCGGTAT TATCCCGTGA
 TGACGCCGGG
 CAAGAGCAAC TCGGTCGCCG CATACACTAT TCTCAGAATG ACTTGGTTGA
 GTACTACCA
 GTCACAGAAA AGCATCTTAC GGATGGCATG ACAGTAAGAG AATTATGCAG
 TGCTGCCATA
 ACCATGAGTG ATAACACTGC GGCCAACTTA CTTCTGACAA CGATCGGAGG
 ACCGAAGGAG
 CTAACCGCTT TTTTGCACAA CATGGGGGAT CATGTAACTC GCCTTGATCG
 TTGGGAACCG
 GAGCTGAATG AAGCCATACC AAACGACGAG CGTGACACCA CGATGCCAGC
 AGCAATGGCA
 ACAACGTTGC GCAAACCTATT AACTGGCGAA CTACTTACTC TAGCTTCCCG

FIG. 5B

GCAACAATTA
ATAGACTGGA TGGAGGCGGA TAAAGTTGCA GGACCACTTC TGCGCTCGGC
CCTTCCGGCT TTGCTGATAA ATCTGGAGCC GGTGAGCGTG GGTCTCGCGG
GGCTGGTTTA CAGATGGTAA GCCCTCCCGT ATCGTAGTTA TCTACACGAC
TATCATTGCA GCAGTGGGGC ATGAACGAAA TAGACAGATC GCTGAGATAG GTGCCTCACT
GGGGAGTCAG CAGACCAAGT TTACTCATAT ATACTTTAGA TTGATTTAAA
GCAACTATGG GGATCTAGGT GAAGATCCTT TTTGATAATC TCATGACCAA
GATTAAGCAT CGTGAGTTTT CGTTCCACTG AGCGTCAGAC CCCGTAGAAA AGATCAAAGG
TGGTAACTGT CGTTCCACTG AGCGTCAGAC CCCGTAGAAA AGATCAAAGG
ACTTCATTTT TTCTGCGCGT AATCTGCTGC TTGCAAACAA AAAAACCACC
TAATTTAAAA TGCCGGATCA AGAGCTACCA ACTCTTTTTC CGAAGGTAAC
AATCCCTTAA TACCAAATAC TGTCTTCTA GTGTAGCCGT AGTTAGGCCA
CGTGAGTTTT CACCGCCTAC ATACCTCGCT CTGCTAATCC TGTTACCAGT
ATCTTCTTGA TACCGGGTTG GACTCAAGAC GATAGTTACC
GATCCTTTTT GCTGAACGGG GGGTTCGTGC ACACAGCCCA GCTTGGAGCG
GCTACCAGCG AGTGGCGATA AGTCGTGTCT TACCGGGTTG GACTCAAGAC GATAGTTACC
GTGGTTTGTT GCTGAACGGG GGGTTCGTGC ACACAGCCCA GCTTGGAGCG
TGGCTTCAGC GATACCTACA GCGTGAGCAT TGAGAAAGCG CCACGCTTCC
AGAGCGCAGA CACCGCCTAC ATACCTCGCT CTGCTAATCC TGTTACCAGT
CCACTTCAAG AGTGGCGATA AGTCGTGTCT TACCGGGTTG GACTCAAGAC GATAGTTACC
AACTCTGTAG GCTGAACGGG GGGTTCGTGC ACACAGCCCA GCTTGGAGCG
GGCTGCTGCC GATACCTACA GCGTGAGCAT TGAGAAAGCG CCACGCTTCC
AGTGGCGATA AGTCGTGTCT TACCGGGTTG GACTCAAGAC GATAGTTACC
GGATAAGGCG GCTGAACGGG GGGTTCGTGC ACACAGCCCA GCTTGGAGCG
CAGCGGTCGG GATACCTACA GCGTGAGCAT TGAGAAAGCG CCACGCTTCC
AACGACCTAC GGTATCCGGT AAGCGGCAGG GTCGGAACAG GAGAGCGCAC
ACCGAACTGA ACGCCTGGTA TCTTTATAGT CCTGTCCGGT TTCGCCACCT
CGAAGGGAGA GTCGATGCTC GTCAGGGGGG CGGAGCCTAT GGAAAAACGC
AAGGCGGACA CTTTGTGCTG CCTTTTGCTC ACATGTTCTT
GAGGGAGCTT GGTTCCTGGC CTTTGTGCTG CCTTTTGCTC ACATGTTCTT
CCAGGGGGAA TGTGATGCTC GTCAGGGGGG CGGAGCCTAT GGAAAAACGC
CTGACTTGAG CTTTGTGCTG CCTTTTGCTC ACATGTTCTT
CGTCGATTTT GGTTCCTGGC CTTTGTGCTG CCTTTTGCTC ACATGTTCTT
CAGCAACGCG CTTTGTGCTG CCTTTTGCTC ACATGTTCTT
GCCTTTTTAC CTGTGGATAA CCGTATTACC GCCTTTGAGT GAGCTGATAC
TCCTGCGTTA GAGCTGATAC
TCCCCTGATT CTGTGGATAA CCGTATTACC GCCTTTGAGT GAGCTGATAC
CGCTCGCCGC
AGCCGAACGA CCGAGCGCAG CGAGTCAGTG AGCGAGGAAG CGGAAGAGC
<end M13>
GCCCAATACGCAA
ACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATCCAGCTGGCACGACAGGTTTCCCGA
CTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTACCTCACTCATTAGGCACC
CCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACA
ATTTACACAGGAAACAGCTATGACCATGATTAC
GAATTAA

FIG. 5C

```

><pCIS.EBON
>< assembled by Steve Williams June 1989
><"poison-minus" pRK
><with EBNA-1, oriP, neoR
><polylinker sites: XhoI, HindIII, NotI

><CMV enhancer/promoter
T
TCGAGCTCGCCCGACATTGATTA
TTG<SpeI>ACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCC
ATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCT
GACCGCCCAACGACCCCGCCATTGACGTCAATAATGACGTATGTTCCC
ATAGTAACGCCAATAGGGACTTTCATTGACGTCAATGGGTGGAGTATTT
ACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTA
CGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCC
CAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATT
AGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGC
GTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGAC
GTCAATGGGAGTTTGTGTTTGGCACCAAAATCAACGGGACTTTCCAAATG
TCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGT
GGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCG
<Begin RNA>
TCAGATCGCCTGG
AGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATC
CAGCCTCCGCGGGCCGGAACGGTGCATTGGAACGCGGATTCCTCCGTGCCA
AGAGTGACGTAAGTACCGCCTATAGAGTCTATAGGCCACCCCTTGGCT
T
><sp6 promoter>
<GGCCACCCCTTGGCTT>CGTTAGAACGCGGCTACAATTAATACATAACC
T T A T G T A T C A T A C A T A C G A T T T A G G T G A C A C T A T A ><sp6 RNA
start>GAATA<ACATCCACTTTGCCTTTC>

ACATCCACTTTGCCTTCTCTCC
ACAGGTGTCCACTCCCAGGTCCAA<PstI-ClaI converter>CTGCA

><cloning linker>CCTCGGTTCT
ATCGATTCTCGA

<EcoRI/klenow>GAATTAATTC

AAGCTTGCGGCCGCAGCTT
GGCCGCCATGGCCC
><sv40 early poly A>AACTTGTTTATTGCAGCTTATAATGGTT
ACAAATAAAGCAATAGCATCACAAATTTACAAATAAAGCATTTTTTTTCACTGCATTCTA
GTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCT
GGATCGATCGG
> < s v 4 0
origin>GAATTAATTCGGCGCAGCACCATGGCCTGAAATAACCTCTGAAAGAGGAACTTGGTTA

<KpnI>GGTACCTTC
TGAGGCGGAAGAACCAGCT
GTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGC
AGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGC

```

FIG. 6A

TCCCCAGCAGGCAGAAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCG
 CCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCAT
 GGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTC
 CAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCCTAGGCTTTTGCAA

<start pUC118>
 AAGCTGTT<HpaI end from SW1>

><delta 3
 <PCR primer seq; PCR product was blunted & ligated in> CACGTGAT
 <this is EcoRV site remnant>

<RI cassette: EBNA-oriP-tk term-NeoR-tk prom>
 <4913 bp EcoRI-Bam from 220_2>
 <EcoRI>
 GAATTCTCAT
 GTTTGACAGC TTATCATCGA TA

><EBNA-1>
 GATCCTCACA GGCCGCACCC AGCTTTTCTT CCGTTGCCCC AGTAGCATCT CTGTCTGGTG
 ACCTTGAAGA GGAAGAGGAG GGGTCCCGAG AATCCCCATC CCTACCGTCC AGCAAAAAGG
 GGGACGAGGA ATTTGAGGCC TGGCTTGAGG CTCAGGACGC AAATCTTGAG GATGTTTCAGC
 GGGAGTTTTC CGGGCTGCGA GTAATTGGTG ATGAGGACGA GGATGGTTCG GAGGATGGGG
 AATTTTCAGA CCTGGATCTG TCTGACAGCG ACCATGAAGG GGATGAGGGT GGGGGGGCTG
 TTGGAGGGGG CAGGAGTCTG CACTCCCTGT ATTCACTGAG CGTCGTCTAA TAAAGATGTC
 TATTGATCTC TTTTAGTGTG AATCATGTCT GACGAGGGGC CAGGTACAGG ACCTGGAAAT
 GGCCTAGGAG AGAAGGGAGA CACATCTGGA CCAGAAGGCT CCGGCGGCAG TGGACCTCAA
 AGAAGAGGGG GTGATAACCA TGGACGAGGA CGGGGAAGAG GACGAGGACG AGGAGGCGGA
 AGACCAGGAG CCCCAGGCGG CTCAGGATCA GGGCCAAGAC ATAGAGATGG TGTCCGGAGA
 CCCCAGAAAC GTCCAAGTTG CATTGGCTGC AAAGGGACCC ACGGTGGAAC AGGAGCAGGA
 GCAGGAGCGG GAGGGGCAGG AGCAGGAGGG GCAGGAGCAG GAGGAGGGGC AGGAGCAGGA
 GGAGGGGCAG GAGGGGCAGG AGGGGCAGGA GGGGCAGGAG CAGGAGGAGG GGCAGGAGCA
 GGAGGAGGGG CAGGAGGGGC AGGAGGGGCA GGAGCAGGAG GAGGGGCAGG AGCAGGAGGA
 GGGGCAGGAG GGGCAGGAGC AGGAGGAGGG GCAGGAGGGG CAGGAGGGGC AGGAGCAGGA
 GGAGGGGCAG GAGCAGGAGG AGGGGCAGGA GGGGCAGGAG CAGGAGGAGG GGCAGGAGGG
 GCAGGAGGGG CAGGAGCAGG AGGAGGGGCA GGAGCAGGAG GGGCAGGAGG GGCAGGAGGG
 GCAGGAGCAG GAGGGGCAGG AGCAGGAGGA GGGGCAGGAG GGGCAGGAGG GGCAGGAGCA
 GGAGGGGCAG GAGCAGGAGG GGCAGGAGCA GGAGGGGCAG GAGCAGGAGG GGCAGGAGGG
 GCAGGAGCAG GAGGGGCAGG AGGGGCAGGA GCAGGAGGGG CAGGAGGGGC AGGAGCAGGA
 GGAGGGGCAG GAGGGGCAGG AGCAGGAGGA GGGGCAGGAG GGGCAGGAGC AGGAGGGGCA
 GGAGGGGCAG GAGCAGGAGG GGCAGGAGGG GCAGGAGCAG GAGGGGCAGG AGGGGCAGGA
 GCAGGAGGAG GGGCAGGAGC AGGAGGGGCA GGAGCAGGAG GTGGAGGCCG GGGTCGAGGA
 GGCAGTGGAG GCCGGGGTCG AGGAGGTAGT GGAGGCCGGG GTCGAGGAGG TAGTGGAGGC
 CGCCGGGGTA GAGGACGTGA AAGAGCCAGG GGGGGAAGTC GTGAAAGAGC CAGGGGGAGA
 GGTCGTGGAC GTGGAGAAAA GAGGCCAGG AGTCCCAGTA GTCAGTCATC ATCATCCGGG
 TCTCCACCGC GCAGGCCCCC TCCAGGTAGA AGGCCATTTT TCCACCCTGT AGGGGAAGCC
 GATTATTTTG AATACCACCA AGAAGGTGGC CCAGATGGTG AGCCTGACGT GCGCCCGGGA
 GCGATAGAGC AGGGCCCCGC AGATGACCCA GGAGAAGGCC CAAGCACTGG ACCCCGGGGT
 CAGGGTGATG GAGGCAGGCG CAAAAAAGGA GGGTGGTTTG GAAAGCATCG TGGTCAAGGA
 GGTTCCAACC CGAAATTTGA GAACATTGCA GAAGGTTTAA GAGCTCTCCT GGCTAGGAGT
 CACGTAGAAA GGAATACCGA CGAAGGAAGT TGGGTCGCCG GTGTGTTCGT ATATGGAGGT
 AGTAAGACCT CCCTTTACAA CCTAAGGCGA GGAAGTGGCC TTGCTATTCC ACAATGTCGT
 CTTACACCAT TGAGTCGTCT CCCCTTTGGA ATGGCCCTTG GACCCGGCCC ACAACCTGGC
 CCGCTAAGGG AGTCCATTGT CTGTTATTTC ATGGTCTTTT TACAAACTCA TATATTTGCT
 GAGGTTTTGA AGGATGCGAT TAAGGACCTT GTTATGACAA AGCCCGCTCC TACCTGCAAT

FIG. 6B


```

ATCAGGGTGA CTGTGTGCAG CTTTGACGAT GGAGTAGATT TGCCTCCCTG GTTTCACCT
ATGGTGGAAG GGGCTGCCGC GGAGGGTGAT GACGGAGATG ACGGAGATGA AGGAGGTGAT
GGAGATGAGG GTGAGGAAGG GCAGGAGTGA TGTAACCTGT TAGGAGACGC CCTCAATCGT
ATTAAAAGCC GTGTATTCCC CCGCACTAAA GAATAAATCC CCAGTAGACA TCATGCGTGC
TGTTGGTGTA TTTCTGGCCA TCTGTCTTGT CACCATTTTC GTCCTCCCA CATGGGGCAA
TTGGGCATAC CCATGTTGTC ACGTCACTCA GCTCCGCGCT CAACACCTTC TCGCGTTGGA
AAACATTAGC GACATTTACC TGGTGAGCAA TCAGACATGC GACGGCTTTA GCCTGGCCTC
CTTAAATTCA CCTAAGAATG GGAGCAACCA
                                ><oriP>GCATGCAGGA AAAGGACAAG CAGCGAAAAT
TCACGCCCCC TTGGGAGGTG GCGGCATATG CAAAGGATAG CACTCCCCT CTACTACTGG
GTATCATATG CTGAC
><family of repeats>
    TGTAT ATGCATGAGG ATAGCATATG CTACCCGGAT ACAGATTAGG
ATAGCATATA CTACCCAGAT ATAGATTAGG ATAGCATATG CTACCCAGAT ATAGATTAGG
ATAGCCTATG CTACCCAGAT ATAAATTAGG ATAGCATATA CTACCCAGAT ATAGATTAGG
ATAGCATATG CTACCCAGAT ATAGATTAGG ATAGCCTATG CTACCCAGAT ATAGATTAGG
ATAGCATATG CTACCCAGAT ATAGATTAGG ATAGCATATG CTATCCAGAT ATTTGGGTAG
TATATGCTAC CCAGATATAA ATTAGGATAG CATATACTAC CCTAATCTCT ATTAGGATAG
CATATGCTAC CCGGATACAG ATTAGGATAG CATATACTAC CCAGATATAG ATTAGGATAG
CATATGCTAC CCAGATATAG ATTAGGATAG CCTATGCTAC CCAGATATAA ATTAGGATAG
CATATACTAC CCAGATATAG ATTAGGATAG CATATGCTAC CCAGATATAG ATTAGGATAG
CCTATGCTAC CCAGATATAG ATTAGGATAG CATATGCTAT CCAGATATTT GGGTAGTATA
TGCTACCC
><end family of repeats>
    AT GGCAACATTA GCCCACCCTG CTCTCAGCGA CCTCGTGAAT ATGAGGACCA
ACAACCCTGT GCTTGGCGCT CAGGCGCAAG TGTGTGTAAT TTGTCCTCCA GATCGCAGCA
ATCGCGCCCC TATCTTGGCC CGCCACCTA CTTATGCAGG TATTCCCCGG GGTGCCATTA
GTGGTTTTGT GGGCAAGTGG TTTGACCGCA GTGGTTAGCG GGGTTACAAT CAGCCAAGTT
ATTACACCCT TATTTTACAG TCCAAAACCG CAGGGCGGCG TGTGGGGGCT GACGCGTGCC
CCCCTCCAC AATTTCAAAA AAAAGAGTGG CCACTTGTCT TTGTTTATGG GCCCCATTGG
CGTGGAGCCC CGTTTAATTT TCGGGGGTGT TAGAGACAAC CAGTGGAGTC CGCTGCTGTC
GGCGTCCACT CTCTTTCCCC TTGTTACAAA TAGAGTGTA CAACATGGTT CACCTGTCTT
GGTCCCTGCC TGGGACACAT CTTAATAACC CCAGTATCAT ATTGCACTAG GATTATGTGT
TGCCCATAGC CATAAATTCG TGTGAGATGG ACATCCAGTC TTTACGGCTT GTCCCCACCC
CATGGATTTT TATTGTAAA GATATTCAGA ATGTTTCATT CCTACACTAG TATTTATTGC
CCAAGGGGTT TGTGAGGGTT ATATTGGTGT CATAGCACAA TGCCACCACT GAACCCCCCG
TCCAAATTTT ATTCTGGGGG CGTCACCTGA AACCTTGTTT TCGAGCACCT CACATACACC
TTACTGTTCA CAACTCAGCA GTTATTCTAT TAGCTAAACG AAGGAGAATG AAGAAGCAGG
CGAAGATTCA GGAGAGTTCA CTGCCCCGCT CTTGATCTTC AGCCACTGCC CTTGTGACTA
AAATGGTTCA CTACCCTCGT GGAATCCTGA CCCCATGTAA ATAAAACCGT GACAGCTCAT
GGGGTGGGAG ATATCGCTGT TCCTTA
                                ><dyad region>GGAC CCTTTTACTA ACCCTAATTC GATAGCATAT
GCTTCCCGTT GGGTAACATA TGCTATTGAA TTAGGGTTAG TCTGGATAGT ATATACTACT
ACCCGGGAAG CATATGCTA
                                ><end dyad>C CCGTTTAGGG TTAACAAGGG GGCCTTATAA AACTATTGCT
TAATGCCCTC TTGAGGGTCC GCTTATCGGT AGCTACACAG GCCCCTCTGA TTGACGTTGG
TGTAGCCTCC CGTAGTCTTC CTGGGCCCCCT GGGAGGTACA TGTCCTCCAG CATTGGTGTA
AGAGCTTCAG CCAAGAGTTA CACATAAAGG CAATGTTGTG TTGCAGTCCA CAGACTGCAA
AGTCTGCTCC AGGATGAAAG CCACTCAGTG TTGGCAAATG TGCACATCCA TTTATAAGGA
TGTCAACTAC AGTCAGAGAA CCCCTTTGTG TTTGGTCCCC CCCCGTGTCA CATGTGGAAC
AGGGCCCAGT TGGCAAGTTG TACCAACCAA CTGAAGGGAT TACATGCACT GCCCC
><end oriP>
                                ><HSV TK TERM 3'END>TGACC
AATACAAAAC AAAAGCGCTC CTCGTACCAG CGAAGAAGGG GCAGAGATGC CGTAGTCAGG

```

FIG. 6C

TTTAGTTCGT CCGGCGGC
><pUC12 SmaI-HaeIII polylinker>
GG G<Bam site is next>

<1.65 kb Bam-EcoRI frag from pkan2.pcr.bam>
GGATCC

><Bam site made by PCR>
GCCAGAAATCCGCGCGGTGGTTTTTGGGGGTCGG
GGGTGTTTGGCAGCCACAGACGCCCCGGTGTTCTGTCTGCTCAGTCCAGTCGTGGACCTGACCCACG
CCAGGCCATCCAAAACCATGGGTCTGTCTGCTCAGTCCAGTCGTGGACCTGACCCACG
CAACGCCCAAAGAATAACCCCCACGAACCATAAACCATTTCCCATGGGGGACCCCGTCC
CTAACCACGCGGGCCCGTGGCTATGGCGGGCTTGCCGCCCCGACGTTGGCTGCGAGCCCT
GGGCCTTCACCCGAACCTTGGGGGTGGGGTGGGGAAAAGGAAGAAACGCGGGCGTATTGG
CCCCAATGGGGTCTCGGTGGGGTATCGACAGAGTGCCAGCCCTGGGACCGAACCCCGCGT
TTATGAACAAACGACCCAACACCCGTGCGTTTTATTCTGTCTTTTATTGCCGTCATAGC
GCGGGTTCCTTCCGGTATTGTCTCCTTCCGTGTTTCAGTTAGCCTCCCCCATCT
<HSV1 tk terminator SmaI>

<following is EcoRI - SmaI from pKan2, rc>
><tn5 neomycin phosphotransferase gene>
<BglII-Sma, rc>

<SmaI, AvaI>CCCGGGGTGGGCGAAGAACTCCAGCATGAGATCCCCGCGCT
GGAGGATCATCCAGCCGGCGTCCCGGAAAACGATTCCGAAGCCCAACCTTTCATAGAAGG
CGGCGGTGGAATCGAAATCTCGTGATGGCAGGTTGGGCGTCGCTTGGTCGGTCATTTCTGA
ACCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGA
ATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTC
TTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCG
GCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCTGGCAAGCAGGC
ATCGCCATGGGTACGACGAGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAA
CAGTTCGGCTGGCGCGAGCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACC
GGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCA
GGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTC
GGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCA
GTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCTGTGGC
CAGCCACGATAGCCGCGCTGCCTCGTCTGTCAGTTCATTTCAGGGCACCGGACAGGTCGGT
CTTGACAAAAGAACCAGGGCGCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCA
GCCGATTGTCTGTTGTGCCAGTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGAGGA
ACCTGCGTGCAATCCATCTTGTTCATCATGCGAAACGATCCTCATCTGTCTCTTGATC

><tk promoter>
<EcoRI-BglII, rc>
<BglII>AGATCTGCGGCACGCTGTTGACGCTGTTAAGCGGGTCGCTGCA
GGGTCTGCTCGGTGTTTCGAGGCCACACGCGTCACCTTAATATGCGAAGTGGACCTGGGACC
GCGCCGCCCCGACTGCATCTGCGTGTTTGAATTC<EcoRI>

<back to SW2 sequence; EcoRV site remnant>
A

><M13 ori>
TCAAAGCAACCATAGTACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTG
TGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTTCG

FIG. 6D

CTTTCTTCCCTTCCTTTCTCGCCACGTTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGG
 GGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAACTTGATT
 TGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGT
 TGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTA
 TCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTTCGGCCTATTGGTTAAAAA
 ATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTT

TATGGTGCA<ApaLI/blunt>

><delta 2a>

			<Eco0109I/blunt>GGCCTCG	TGATACGCCT
ATTTTTATAG				
GTAAATGTCA	TGATAATAAT	GGTTTCTTAG	ACGTCAGGTG	GCACTTTTCG
GGGAAATGTG				
CGCGGAACCC	CTATTTGTTT	ATTTTTCTAA	ATACATTCAA	ATATGTATCC
GCTCATGAGA				
CAATAACCCT	GATAAATGCT	TCAATAATAT	TGAAAAAGGA	AGAGTATGAG
TATTCAACAT				
TTCCGTGTCTG	CCCTTATTCC	CTTTTTTGCG	GCATTTTGCC	TTCTGTTTTT
TGCTCACCCA				
GAAACGCTGG	TGAAAGTAAA	AGATGCTGAA	GATCAGTTGG	GTGCACGAGT
GGGTTACATC				
GAACGGATC	TCAACAGCGG	TAAGATCCTT	GAGAGTTTTC	GCCCCGAAGA
ACGTTTTCCA				
ATGATGAGCA	CTTTTAAAGT	TCTGCTATGT	GGCGCGGTAT	TATCCCGTGA
TGACGCCGGG				
CAAGAGCAAC	TCGGTCGCCG	CATACACTAT	TCTCAGAATG	ACTTGGTTGA
GTA CTCACCA				
GTCACAGAAA	AGCATCTTAC	GGATGGCATG	ACAGTAAGAG	AATTATGCAG
TGCTGCCATA				
ACCATGAGTG	ATAACACTGC	GGCCAACCTA	CTTCTGACAA	CGATCGGAGG
ACCGAAGGAG				
CTAACCGCTT	TTTTGCACAA	CATGGGGGAT	CATGTAACTC	GCCTTGATCG
TTGGGAACCG				
GAGCTGAATG	AAGCCATACC	AAACGACGAG	CGTGACACCA	CGATGCCAGC
AGCAATGGCA				
ACAACGTTGC	GCAAACCTATT	AACTGGCGAA	CTACTTACTC	TAGCTTCCCG
GCAACAATTA				
ATAGACTGGA	TGGAGGCGGA	TAAAGTTGCA	GGACCACTTC	TGCGCTCGGC
CCTTCCGGCT				
GGCTGGTTTA	TTGCTGATAA	ATCTGGAGCC	GGTGAGCGTG	GGTCTCGCGG
TATCATTGCA				
GCACTGGGGC	CAGATGGTAA	GCCCTCCCGT	ATCGTAGTTA	TCTACACGAC
GGGGAGTCAG				
GCAACTATGG	ATGAACGAAA	TAGACAGATC	GCTGAGATAG	GTGCCTCACT
GATTAAGCAT				
TGGTAACTGT	CAGACCAAGT	TTACTCATAT	ATACTTTAGA	TTGATTTAAA
ACTTCATTTT				
TAATTTAAAA	GGATCTAGGT	GAAGATCCTT	TTTGATAATC	TCATGACCAA
AATCCCTTAA				
CGTGAGTTTT	CGTTCCACTG	AGCGTCAGAC	CCCGTAGAAA	AGATCAAAGG
ATCTTCTTGA				
GATCCTTTTT	TTCTGCGCGT	AATCTGCTGC	TTGCAAACAA	AAAAACCACC

FIG. 6E

```
GCTACCAGCG
      GTGGTTTGTT  TGCCGGATCA  AGAGCTACCA  ACTCTTTTTC  CGAAGGTAAC
TGGCTTCAGC
      AGAGCGCAGA  TACCAAATAC  TGTCTTCTA  GTGTAGCCGT  AGTTAGGCCA
CCTACTCAAG
      AACTCTGTAG  CACCGCCTAC  ATACCTCGCT  CTGCTAATCC  TGTTACCAGT
GGCTGCTGCC
      AGTGGCGATA  AGTCGTGTCT  TACCGGGTTG  GACTCAAGAC  GATAGTTACC
GGATAAGGCG
      CAGCGGTCGG  GCTGAACGGG  GGGTTCGTGC  ACACAGCCCA  GCTTGGAGCG
AACGACCTAC
      ACCGAACTGA  GATACCTACA  GCGTGAGCAT  TGAGAAAGCG  CCACGCTTCC
CGAAGGGAGA
      AAGGCGGACA  GGTATCCGGT  AAGCGGCAGG  GTCGGAACAG  GAGAGCGCAC
GAGGGAGCTT
      CCAGGGGGAA  ACGCCTGGTA  TCTTTATAGT  CCTGTCGGGT  TTCGCCACCT
CTGACTTGAG
      CGTCGATTTT  TGTGATGCTC  GTCAGGGGGG  CGGAGCCTAT  GGAAAAACGC  CAG
```

><delta1.PVU>

<PvuII site introduced by mutagenesis; 228 bp PvuII fragment deleted>

<join to PvuII at 4532 in RK5>

```
CTGGCAGCAGAGGTTTCCCGA
CTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTACCTCACTCATTAGGCACC
CCAGGCTTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACA
ATTTACACAGGAAACAGCTATGACCATGATTAC
GAATTAA
```

FIG. 6F

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/04648

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/19; C07K13/00; A61K37/02		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C07K ; C12N ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,9 205 184 (THE UNITED STATES OF AMERICA) 2 April 1992 see page 4, line 1 - page 6, line 6 see page 10, line 22 - page 11, line 14 see page 13, line 7 - page 14, line 11 ---	1,2, 37-41, 46-55
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 172, no. 1, 15 October 1990, DULUTH, MINNESOTA US pages 321 - 327 TATSUYA, SEKI ET AL. 'Isolation and expression of cDNA for different forms of hepatocyte growth factor from human leukocyte' cited in the application see page 322, paragraph 1; figure 2 see page 326, paragraph 1 ---	16,35, 41-43, 49-53
-/--		
¹⁰ Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "A" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 27 AUGUST 1993		Date of Mailing of this International Search Report 23. 09. 93
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer MONTERO LOPEZ B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claims No.
A	<p>BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 180, no. 2, 31 October 1991, DULUTH, MINNESOTA US pages 1151 - 1158 NOBUYUKI SHIMA ET AL. 'Tumor cytotoxic factor / hepatocyte growth factor from human fibroblasts: cloning of its cDNA, purification and characterization of recombinant protein' see abstract see page 1151, paragraph 1 - page 1152, paragraph 1; figure 1 see page 1154, paragraph 2 - page 1157, paragraph 1</p> <p>---</p>	<p>16, 35, 41-43, 49-53</p>
P, X	<p>EMBO JOURNAL vol. 11, no. 7, July 1992, EYNSHAM, OXFORD GB pages 2503 - 2510 NATHALIE A. LOKKER ET AL. 'Structure-function analysis of hepatocyte growth factor: identification of variants that lack mitogenic activity yet retain high affinity receptor binding' see abstract see page 2505, left column, paragraph 3 - page 2508, right column, paragraph 1</p> <p>---</p>	<p>1-7, 9-12, 15-41, 46-53</p>
P, X	<p>JOURNAL OF BIOLOGICAL CHEMISTRY vol. 267, no. 28, 5 October 1992, BALTIMORE, MD US pages 20114 - 20119 DAIJI NAKA ET AL. 'Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer' see abstract see page 20114, left column, paragraph 2 - right column, paragraph 2 see page 20116, right column, paragraph 2 - page 20118, right column, paragraph 2; figure 4</p> <p>---</p> <p>--- -/--</p>	<p>1-7, 9, 11, 12, 15-17, 36, 37, 49-53</p>

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P, X	<p>FEBS LETTERS. vol. 311, no. 1, October 1992, AMSTERDAM NL pages 17 - 21 EVA GAK ET AL. 'Processing of hepatocyte growth factor to the heterodimeric form is required for biological activity' see page 17, left column, paragraph 2 - right column, paragraph 1 see page 18, right column, paragraph 2 - page 20, left column, paragraph 1 -----</p>	<p>1-7, 9-12, 15, 36-39, 49-53</p>

INTERNATIONAL SEARCH REPORT

I. International application No.

PCT/US 93/ 04648

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 55 is directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

US 9304648
SA 74139

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 27/08/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9205184	02-04-92	AU-A- 8659591	15-04-92
		CA-A- 2091700	15-03-92

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